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Robo3 isoforms have distinct roles during zebrafish development

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Abstract

Roundabout (Robo) receptors and their secreted ligand Slits have been shown to function in a number of developmental events both inside and outside of the nervous system. We previously cloned zebrafish *robo* orthologs to gain a better understanding of Robo function in vertebrates. Further characterization of one of these orthologs, *robo3*, has unveiled the presence of two distinct isoforms, *robo3 variant 1* (*robo3var1*) and *robo3 variant 2* (*robo3var2*). These two isoforms differ only in their 5′-ends with *robo3var1*, but not *robo3var2*, containing a canonical signal sequence. Despite this difference, both forms accumulate on the cell surface. Both isoforms are contributed maternally and exhibit unique and dynamic gene expression patterns during development. Functional analysis of *robo3* isoforms using an antisense gene knockdown strategy suggests that Robo3var1 functions in motor axon pathfinding, whereas Robo3var2 appears to function in dorsoventral cell fate specification. This study reveals a novel function for Robo receptors in specifying ventral cell fates during vertebrate development.

Keywords: Dorsoventral patterning; Signal sequence; Morpholinos; Motor axons

1. Introduction

The Roundabout (Robo) family of receptors and their extracellular ligands, the Slit protein family, were initially identified by their evolutionarily conserved role in repulsive axon guidance in the developing central nervous system (Brose et al., 1999; Brose and Tessier-Lavigne, 2000). More recently, this signaling pathway has been implicated in diverse developmental processes outside of the nervous system, including endothelial cell migration, regulation of leukocyte migration, and lung development (Wu et al., 2001; Xian et al., 2001; Park et al., 2003). In addition, different roles in tumorigenesis have been described. Slit2 has been demonstrated to encode tumor suppressor activity in gliomas, lung and breast cancers (Dallol et al., 2002, 2003). Conversely, Slit2 and Robo1 are important for promoting angiogenesis of solid tumors (Wang et al., 2003).

Four zebrafish Slit orthologs, *slit1a*, *slit1b*, *slit2* and *slit3*, have been identified (Hutson et al., 2003; Yeo et al., 2001). *slit2* and *slit3* mRNA expression is observed in the midline axial mesoderm during mid-blastula and gastrula stages in addition to their dynamic expression patterns during later developmental stages (Yeo et al., 2001). Overexpression of *slit2* mRNA throughout the early zebrafish embryo results in a broadening of the neural anlagen, broadening and shortening of the chordamesoderm, and positioning of the prechordal mesoderm posteriorly (Yeo et al., 2001). These results were interpreted as defects in convergent and extension cell movements during gastrulation.

Four Robo receptors (1–4) have been identified in zebrafish (Lee et al., 2001; Challa et al., 2001; Park et al., 2003). This is the same number of Robo family members found in other higher vertebrates, suggesting that this gene family has not undergone duplications in the zebrafish lineage. Robo2 functions in retinal tectal axon guidance as evidenced by the *robo2/astray* mutant (Fricke et al., 2001) and in kidney formation (Grieshammer et al., 2004).

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Robo4, which is a highly divergent Robo family member in vertebrates, functions in endothelial migration and angiogenesis (Bedell et al., 2005; Huminiecki et al., 2002; Park et al., 2003).

In the current study, we describe two isoforms of the Robo3 ortholog in zebrafish and report their unique roles in development. Knockdown analysis reveals that one of the isoforms is involved in early dorsal ventral patterning, whereas the other functions either directly or indirectly in motor axon guidance. This study provides further evidence for multiple roles played by Robo family members during vertebrate development.

2. Results

2.1. Zebrafish robo3 isoforms

We have previously described the identification and initial characterization of two zebrafish *robo* orthologs, *robo1* and *robo3* (Challa et al., 2001). Further analysis of the *robo3* ortholog by 5′ RACE PCR using total RNA from 24 to 36 hours post-fertilization (hpf) embryos revealed that there were at least two distinct species. DNA sequencing of the two species provided evidence for the existence of two isoforms of *robo3*. The two isoforms were identical in the sequence encoding the core protein but diverged at the 5′ end, which included the 5′ UTR and sequences encoding a short stretch of amino acids corresponding to the signal sequence; we named these isoforms *robo3 variant 1* (*robo3var1*) and *robo3 variant 2* (*robo3var2*; Genebank Accession No. AF337036 and AF304131, respectively). While the *robo3var2* sequence was the same as the one described earlier (Challa et al., 2001), the *robo3var1* sequence was identical to that which was described by Lee et al. (2001). To further ascertain the presence of both isoforms, we performed RT-PCR experiments using isoform specific forward primers and common reverse primers and found that both isoforms were expressed in embryos (data not shown).

Using the Sanger Institute zebrafish sequencing project (http://www.sanger.ac.uk/) and comparing the genomic and cDNA sequences, we analyzed the genomic organization of the *robo3* locus. Our earlier studies using the LN54 Radiation Hybrid panel (Hukriede et al., 1999) mapped *robo3* to Chromosome (Chr) 10 (Challa et al., 2001); Lee et al. (2001) also reported that *robo3* mapped to Chr 10. Genomic sequence analysis using data from the ENSEMBL project, and the ENSEMBL predictions confirm these observations [*robo3var2* (ENSDART00000023575); *robo3var1* (ENSDART00000024778)]. The *robo3* locus is located in the Zv4_scaffold900, flanked by ESTs fc17e04 and fa16f09.s1. Sequence analysis indicates that exon 1 is unique to *robo3var2* and exon 2 is unique to *robo3var1* and the remaining exons are identical. Robo3var2 contains a large first intron of 257.91 kb that does not appear to encode any additional transcripts. Thus, both RNA and genomic analysis substantiate that these variants represent two isoforms of Robo3.

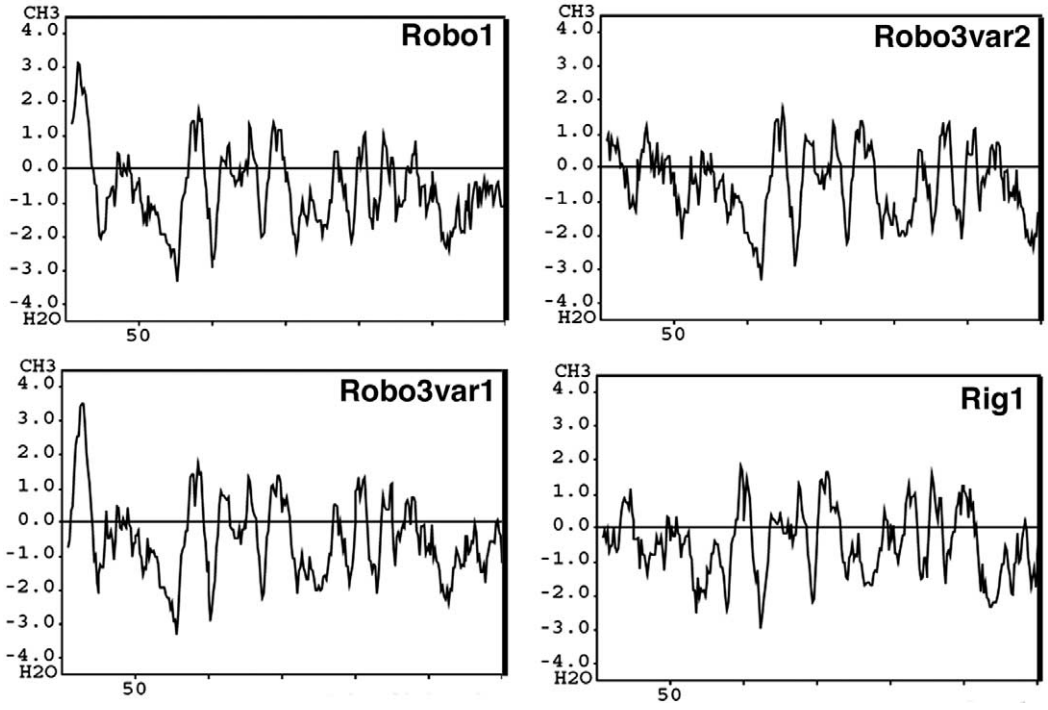

Fig. 1. Computational analysis of Robo proteins. Kyte–Doolittle hydropathy plots for the first 300 amino acids (*x*-axis) of zebrafish Robo1, Robo3var2, Robo3var1, and Mouse Rig-1/Robo3 proteins. On the *y*-axis, positive numbers are more hydrophobic and negative numbers are less hydrophobic.