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15-Zinc finger protein Bloody Fingers is required for zebrafish morphogenetic movements during neurulation

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Abstract

A novel zebrafish gene *bloody fingers (blf)* encoding a 478 amino acid protein containing fifteen C_2H_2 type zinc fingers was identified by expression screening. As determined by in situ hybridization, *blf* RNA displays strong ubiquitous early zygotic expression, while during late gastrulation and early somitogenesis, *blf* expression becomes transiently restricted to the posterior dorsal and lateral mesoderm. During later somitogenesis, *blf* expression appears only in hematopoietic cells. It is completely eliminated in *cloche, moonshine* but not in *vlad tepes (gata1)* mutant embryos. Morpholino (MO) knockdown of the Blf protein results in the defects of morphogenetic movements. Blf-MO-injected embryos (morphants) display shortened and widened axial tissues due to defective convergent extension. Unlike other convergent extension mutants, *blf* morphants display a split neural tube, resulting in a phenotype similar to the human open neural tube defect spina bifida. In addition, dorsal ectodermal cells delaminate in *blf* morphants during late somitogenesis. We propose a model explaining the role of *blf* in convergent extension and neurulation. We conclude that *blf* plays an important role in regulating morphogenetic movements during gastrulation and neurulation while its role in hematopoiesis may be redundant.

Keywords: Zebrafish; Convergent extension; Neurulation; Zinc finger; Gastrulation; Neural tube; Spina bifida; Blood; Hematopoietic

Introduction

Morphogenetic movements accompanied by coordinated cell migration and cell shape changes lead to the establishment of the body axis during vertebrate development. During zebrafish gastrulation, prospective mesendodermal cells involute and ingress. This is followed by convergence and extension movements in which mesendoderm and ectoderm undergo cell intercalations along the medial–lateral axis that narrow the tissues and consequently extend them along the anterior–posterior axis (reviewed in Heisenberg and Tada, 2002). Gastrulation is followed by neurulation during which the neural plate is specified and transformed into neural tube. In contrast to other vertebrates, neural folds are not evident in zebrafish; instead, a neural keel is formed (reviewed in Strahle and Blader, 1994). The neural tube is then shaped by oriented cell divisions and convergent extension (CE) movements. It has been proposed that the mechanism of teleost neurulation is similar to the secondary neurulation seen in the tailbud of higher vertebrates. However, recent studies have demonstrated the epithelial origin of the zebrafish neural keel, arguing that zebrafish neural tube formation may be just a variant of primary neurulation (Papan and Campos-Ortega, 1994; Reichenbach et al., 1990; reviewed in Lowery and Sive, 2004).

Several zebrafish mutants have been identified which exhibit defects in convergent extension. Among them are *pipetail (ppt)*, *knypek (kny)*, and *trilobite (tri)* mutant embryos which exhibit a shortened body axis from late gastrulation stages onwards (Hammerschmidt et al., 1996; Marlow et al., 1998; Solnica-Krezel et al., 1996). These loci have been shown to encode *wnt5*, *glypican knypek*, and *strabismus*, respectively, components or modulators of the

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wnt non-canonical signaling pathway (Jessen et al., 2002; Rauch et al., 1997; Topczewski et al., 2001). This pathway is analogous to the planar cell polarity (PCP) pathway in *Drosophila* and is thought to regulate convergent extension movements in vertebrates (Darken et al., 2002; Heisenberg et al., 2000; Park and Moon, 2002; Shulman et al., 1998; Wallingford et al., 2000). Some other transducers or modulators of the PCP pathway have also been shown to affect convergent extension in zebrafish including Frizzled-2 (Fz2) and Prickle (Pk) proteins (Carreira-Barbosa et al., 2003; Kilian et al., 2003; Sumanas et al., 2001; Veeman et al., 2003).

Defects in convergent extension and neurulation are known to cause embryo abnormalities in many vertebrates, including humans. Incomplete closure of the neural tube, spina bifida, is among the most common birth defects contributing to infant mortality and serious disability (Copp et al., 2003). Mutations in the mouse orthologs of the *Drosophila* PCP genes *disheveled*, *strabismus*, *scribble*, and *flamingo*, all result in failure to initiate neural tube closure (Ueno and Greene, 2003). Loss of function of *wnt*, *frizzled-7*, *disheveled*, *strabismus*, and *prickle* has been shown to perturb gastrulation movements and result in open neural tube in *Xenopus* (Hoppler et al., 1996; Goto and Keller, 2002; Sumanas et al., 2000; Takeuchi et al., 2003; Wallingford and Harland, 2002; Winklbauer et al., 2001). These data demonstrate the involvement of the PCP pathway in regulating both convergent extension and neurulation and illustrates the interdependence of both fundamental processes of morphogenesis in different vertebrates. However, the open neural tube phenotype has not been described in the zebrafish as yet.

In the current study, we have identified a gene encoding a novel zebrafish multiple C_2H_2 zinc finger protein family member, *Bloody fingers (Blf)*. Zinc finger proteins contain a small peptide domain with a special secondary structure stabilized by a zinc ion bound to Cys and His residues of the finger (reviewed in Iuchi, 2001). The most common type of zinc fingers, C_2H_2, is primarily involved in DNA binding and transcriptional regulation. There are hundreds of different C_2H_2 proteins in vertebrate genomes. One of the subclasses of C_2H_2 proteins includes multiple-adjacent C_2H_2 zinc finger proteins. Members of this subclass can have as many as 29 adjacent zinc fingers such as in the protein Roaz (Tsai and Reed, 1997). In addition to DNA binding, zinc fingers in these proteins can also engage in protein–protein interactions to promote homo- or hetero-dimerization such as the one observed between the blood-specific Ikaros protein and two of its homologues, Alios and Helios (Kelley et al., 1998; Morgan et al., 1997). Some of the multiple C_2H_2 domain proteins are also known to bind single-stranded or double-stranded RNA, e.g., TFIIIA and dsRBP-Zfa proteins (Finerty and Bass, 1997; Friesen and Darby, 1998).

Blf protein is predicted to contain 15 sequential zinc fingers spanning almost the entire length of the protein with no other motives detectable. *Blf* RNA is expressed ubiquitously in early zebrafish embryos and is progressively restricted to the posterior dorsal and lateral mesoderm during gastrulation while later expressed in the hematopoietic progenitor cells. Knockdown of Blf protein using antisense morpholino oligonucleotides (MOs) revealed its critical role in convergent extension and neurulation movements. Blf-MO-injected embryos display shortened body axis, split neural tube similar to the spina bifida condition in humans, and delaminating dorsal ectodermal cells. This is the first time that a phenotype similar to spina bifida has been demonstrated in zebrafish suggesting that zebrafish can be used to model this human birth defect. We also propose a model explaining how defective convergent extension results in the open neural tube of *blf* morphants.

Materials and methods

Blf clone isolation

A cDNA clone of 1.92 kb encompassing a short stretch of the 5′UTR, the complete putative ORF sequence of *blf* and the 3′UTR was isolated from the zebrafish embryonic blood cell-specific cDNA library (Long et al., 2000). Using the 5′RACE kit (Promega), an additional 5′UTR sequence was isolated from synthesized cDNA derived from total purified RNA of 24 hpf zebrafish embryos. The combined size of the isolated *blf* cDNA and a predicted poly-A tail of 200–300 nucleotides is in close agreement with the experimentally observed *blf* size of ~2.4 kb, as determined by Northern blotting, suggesting that the complete *blf* cDNA sequence was isolated.

In situ hybridization

In situ hybridization was performed as described (Jowett, 1999). To synthesize DIG-labeled antisense *blf* probe, a *blf*-pTriplEx (Clontech) construct was linearized with *Kpn*I and transcribed with T7 RNA polymerase (Promega). Other probes used include: *no tail (ntl)* (Schulte-Merker et al., 1994); *sonic hedgehog (shh)* (Ekker et al., 1995); *spondin-1b (spon1b)* (Higashijima et al., 1997); *myod* (Weinberg et al., 1996); *crestin* (Luo et al., 2001); cb497 cDNA, corresponding to *hairy-related 4 (her4)* (Thisse et al., 2001); cb112 cDNA, similar to *cytokeratin E7* (Thisse et al., 2001); *gata1* (Detrich et al., 1995); *gata2* (Detrich et al., 1995); *tal1 (scl)* (Liao et al., 1998); *cmyb* (Thompson et al., 1998); and *ikaros* (Willett et al., 2001). To synthesize a probe for the *forkhead box A1 (foxa1)*, the ORF of *foxa1* was amplified by PCR from the post-somitogenesis stage cDNA library (kindly donated by S. C. Ekker), subcloned into the 4-TOPO vector (Invitrogen), linearized with *Spe*I, and transcribed using T7 RNA polymerase.