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The cell adhesion-associated protein Git2 regulates morphogenetic movements during zebrafish embryonic development

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ABSTRACT

Signaling through cell adhesion complexes plays a critical role in coordinating cytoskeletal remodeling necessary for efficient cell migration. During embryonic development, normal morphogenesis depends on a series of concerted cell movements; but the roles of cell adhesion signaling during these movements are poorly understood. The transparent zebrafish embryo provides an excellent system to study cell migration during development. Here, we have identified zebrafish *git2a* and *git2b*, two new members of the *GIT* family of genes that encode ArfGAP proteins associated with cell adhesions. Loss-of-function studies revealed an essential role for Git2a in zebrafish cell movements during gastrulation. Time-lapse microscopy analysis demonstrated that antisense depletion of Git2a greatly reduced or arrested cell migration towards the vegetal pole of the embryo. These defects were rescued by expression of chicken GIT2, indicating a specific and conserved role for Git2 in controlling embryonic cell movements. Git2a knockdown embryos showed defects in cell morphology that were associated with reduced cell contractility. We show that Git2a is required for phosphorylation of myosin light chain (MLC), which regulates myosin II-mediated cell contractility. Consistent with this, embryos treated with Blebbistatin-a small molecule inhibitor for myosin II activity-exhibited cell movement defects similar to *git2a* knockdown embryos. These observations provide *in vivo* evidence of a physiologic role for Git2a in regulating cell morphogenesis and directed cell migration via myosin II activation during zebrafish embryonic development.

Introduction

The precise regulation of cell adhesion signaling to coordinate cytoskeleton organization is essential for tissue morphogenesis and cell migration (Gumbiner, 2005; Hynes, 2009). Although extensively studied in cell culture systems, the functional role of adhesion-associated proteins in regulating cell morphogenesis and migration during embryonic development is poorly understood. The zebrafish (*Danio rerio*), with the powerful advantages of tractable genetics and transparent embryos, has rapidly emerged as an excellent animal model to dissect the molecular complexity of these dynamic cell behaviors.

Morphogenetic cell movements, including epiboly, involution and convergent extension, are fundamental for formation of the primary germ layers during embryonic development (Warga and Kimmel, 1990). During zebrafish gastrulation, blastoderm cells involute and migrate towards the animal pole of the embryo to form the hypoblast cell layer that will give rise to mesoderm and endoderm. Simultaneously, overlying epiblast cells and enveloping layer (EVL) cells migrate towards the vegetal pole to enclose the yolk cell during a process known as epiboly. The yolk syncytial layer (YSL), which lies under the blastoderm in the yolk cell, also undergoes epiboly and is thought to influence EVL epiboly movements. However, mechanisms that control epiboly are not well understood.

Differential cell–cell and cell–extracellular matrix (ECM) adhesion, in concert with the development of tension within the cell cortical cytoskeleton are proposed to be the major driving forces for cell migration *in vivo* (Kane et al., 2005; Köppen et al., 2006; Steinberg, 2007; Latimer and Jessen, 2010). Furthermore, directional cell migration during zebrafish gastrulation is coordinated by the distribution of ECM proteins, such as fibronectin (Latimer and Jessen, 2010), and chemotactic gradients of soluble factors, such as PDGF and S1P (Montero et al., 2003; Kai et al., 2008). In addition, the integrity of E-cadherin based cell–cell junctions (Kane et al., 2005) and signaling via a number of cell–ECM adhesion-associated components including integrin beta1, laminin, FAK, paxillin (Crawford et al., 2003), ILK and parvins (Postel et al., 2008) play critical roles. Finally, downstream activation of the Rho family of GTPases culminates in the development of actomyosin-based contractility and cytoskeleton remodeling that is necessary for directed cell migration (Köppen et al., 2006; Krieg et al., 2008; Weiser et al., 2009).

Abbreviations: ArfGAP, Arf GTPase activating protein; EVL, enveloping layer; ECM, Extracellular matrix; FAK, focal adhesion kinase; PAK, p21-activated kinase; PIX, PAK interacting exchange factor.

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In mammalian cells, the GIT family of Arf GAP (GTPase activating protein) proteins perform an important role in the coordination of Rho GTPase signaling and thus cell motility through their ability to interact with the focal adhesion adaptor paxillin as well as the Rac1/Cdc42 GEF (guanine exchange factor) PIX and the effector p21-activated kinase (PAK) at sites of cell adhesion (Turner et al., 1999). GIT proteins are substrates for non-receptor tyrosine kinases FAK and Src. GIT2 tyrosine phosphorylation is necessary for directing cell migration upon PDGF growth factor signaling in fibroblasts (Brown et al., 2005; Yu et al., 2009), while GIT1 is similarly required for EGF-dependent vascular smooth muscle cell migration (Yin et al., 2005). Gene ablation in mice suggests differential physiological roles for GIT1 and GIT2, with GIT1 playing a critical role in lung and vasculature development (Pang et al., 2009) and GIT2 being important for neutrophil chemotaxis in association with the immune response (Mazaki et al., 2006). Interestingly, mutation of GIT in invertebrates also results in significant developmental defects such as impaired myotube guidance in *Drosophila melanogaster* (Bahri et al., 2009) and deregulated gonad distal tip cell migration in *C. elegans* (Lucanic and Cheng, 2008). However, it is currently unclear how GIT proteins regulate cell motility *in vivo* during vertebrate development.

Herein, we use zebrafish as an animal model to evaluate the physiological importance of GIT2 during embryo development. We have identified two zebrafish GIT2 genes, *git2a* and *git2b*, that are ubiquitously expressed in the early embryo. Taking a reverse genetic approach, we have found that Git2a plays an essential role in regulating morphogenetic cell movements during early embryo development. We show Git2a is required for normal epiboly of EVL and epiblast cells, and use time-lapse imaging of epiblast cells to demonstrate that Git2a controls directional cell migration during epiboly. In addition, we show that Git2a is required for the phosphorylation (activation) of myosin II in EVL cells during epiboly, implicating Git2 in the control of actomyosin based cortical actin contractility in cells undergoing epiboly movements.

Results

Identification of two Git2 orthologs in the zebrafish genome

ArfGAP GIT proteins have been implicated in the regulation of cell adhesion and motility in mammalian cell culture systems and knockout mice (Frank, et al., 2006; Mazaki et al., 2006; Pang et al., 2009; Yu et al., 2009). To examine the role of GIT in modulating dynamic cell behaviors *in vivo*, we chose to use the transparent zebrafish embryo as a model, which is ideal for live imaging studies. By searching the zebrafish genome database, we identified two genes highly similar to mammalian *GIT2*. We have designated these genes as *git2a*, which is located on chromosome 5 (NCBI GenBank Accession: CAM15570), and *git2b*, located on chromosome 10 (NCBI GenBank Accession: AAI29228). Phylogenetic analysis suggested that the zebrafish *git2* genes encode proteins that are evolutionally conserved, especially among vertebrates (Fig. 1A and Sup. Figs. S1A, B). Cloning and sequencing of zebrafish *git2a* cDNA showed 70% nucleic acid identity and 75% amino acid identity with human GIT2 (Sup. Fig. S1B). Amino acid alignment and domain analysis suggest that zebrafish Git2 proteins are highly conserved within major functional domains characterized in other species, including an ArfGAP domain at the N-terminus, three Ankyrin repeats, two Spa2 homology domains, and a paxillin binding subdomain at the C-terminus (Sup. Fig. S1C). Interestingly, phosphorylated tyrosine residues 286, 392 and 592 identified in chicken and mammalian GIT2 proteins (Brown et al., 2005) are highly conserved in zebrafish Git2 proteins (Y296, Y402, Y583), but not in invertebrates (worm and fly) (Sup. Figs. S1A,C).

Characterization of zebrafish Git2 expression during early development

To characterize expression of *git2* genes in the early zebrafish embryo, we first performed whole-mount RNA *in situ* hybridizations. Using antisense *git2a* probes, we detected maternal *git2a* transcripts at the 4-cell stage, and ubiquitous expression during epiboly, throughout somitogenesis and at 24 hours post-fertilization (hpf) (Fig. 1B). Similar maternal and ubiquitous expression of *git2b* was observed during the first 24 hours of zebrafish development (Sup. Figs. S2A,C,E,F,G). Control sense probes showed greatly reduced or absent staining (Sup. Figs. S2B,D), and RT-PCR was used to further confirm maternal expression of *git2a* and *git2b* (data not shown).

Western blotting was performed to evaluate the protein expression profile of Git2 during early development. In wild-type embryos, Git2 was expressed at relatively high levels at the dome stage, and then exhibited a modest decrease between 50%-90% epiboly before rebounding during early somitogenesis (Fig. 1C), indicating Git2 expression is differentially regulated during development. We also observed dynamic expression of Paxillin, a Git2 binding protein, which exhibited a modest increase in expression between 75%-90% epiboly (Fig. 1C), in agreement with a previous study (Crawford et al., 2003). The loading control α-tubulin was equal at these stages (Fig. 1C).

Whole-mount immunofluorescent staining using Git2 antibodies showed ubiquitous Git2 protein expression during epiboly stages (Fig. 1D), consistent with ubiquitous mRNA expression (Fig. 1B). Close examination suggests that Git2 localizes at the proximity of cell–cell junctions of EVL cells and in deep cells during epiboly (Fig. 1D). At 24 hpf, Git2 was ubiquitously expressed with enrichment at somite boundaries (Fig. 1E), a region enriched with ECM proteins including fibronectin and laminin, and integrins and associated adhesion proteins such as FAK, paxillin, parvin and ILK (Crawford et al., 2003; Postel et al., 2008). The early expression of Git2 throughout the embryo suggested a functional role for Git2 during early embryonic development.

Git2a knockdown disrupts zebrafish development during gastrulation

In an effort to identify physiologic functions of Git2 *in vivo*, we took a loss-of-function approach to assess the role of Git2a during zebrafish development. Antisense morpholino oligonucleotides (MO) were designed to bind the intron 2–exon 3 splice acceptor site to disrupt splicing of *git2a* transcripts (*git2a* MO) and thereby reduce Git2a protein expression. Injection of *git2a* MO at the 1–2 cell stages resulted in severe developmental defects or lethality by 24 hpf (Fig. 2A). At 48 hpf, *git2a* morphant embryos that survived showed variable defects, including a shortened anterior-posterior axis, kinked tail and edema (Fig. 2A). These defects were not observed in control MO injected embryos (Fig. 2A). To further characterize *git2a* morphants, we examined phenotypes at early embryonic stages using time-lapse microscopy. The majority of *git2a* morphant embryos displayed no obvious morphological abnormalities at 6 hpf prior to gastrulation (Fig. 2B). However, between 6 and 8 hpf (60–75% epiboly stages)–correlating with the onset of gastrulation–*git2a* morphants developed dose-dependent defects in the process of epiboly (Fig. 2B; Figs. S3A–B), during which cells move towards the vegetal pole of the embryo. At 9 hpf, the majority of *git2a* morphants (65.2%; n = 200) showed epiboly defects (Fig. 2C). Abnormalities included a constricted blastoderm margin (Fig. 2B) and a delay or arrest in development (Fig. 2D) that in many cases led to embryo lethality (Sup. Movie 1). We observed a strong correlation between the severity of epiboly defects and embryonic lethality. Injection of *git2a* MO into the yolk cell at the sphere stage to restrict MO to the yolk (Köppen et al., 2006) did not recapitulate these epiboly defects (Fig. 2C), indicating Git2a does not play a major role in the yolk cell during epiboly. By 12 hpf, when control embryos had progressed to