The zebrafish band 4.1 member Mir is involved in cell movements associated with gastrulation

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Abstract

Cellular processes rely on dynamic events occurring between the cortical cytoskeleton and plasma membrane. Members of the Band 4.1 superfamily, which are best known for their ability to tether the cytoskeleton to the plasma membrane, play prominent structural and regulatory roles that influence cell–cell and cell–substrate interactions, endo- and exocytosis, cell polarity, migration, proliferation, and differentiation. We have identified a new member of the zebrafish Band 4.1 superfamily, which is the homolog of human myosin regulatory light chain interacting protein (MIR), and have examined its role in embryonic development. Zebrafish Mir contains the conserved amino-terminal plasma membrane-binding FERM (Band 4.1/ezrin/radixin/moesin) domain as well as other putative protein–protein interacting domains, including a RING finger. Overall, zebrafish Mir is 71% identical to human MIR located at chromosome 6p23-p22.3, and maps on linkage group 19 to a region of synteny with human chromosome 6. In situ hybridization and RT-PCR revealed that mir is expressed maternally and ubiquitously throughout development. Blocking Mir translation using a mir-specific, morpholino-based, knock-down strategy or expressing Mir constructs lacking the RING finger domain disrupts gastrulation and leads to subsequent trunk and tail defects. In severe cases, morphants exogastrulate. The synergistic effect seen when two mir-specific morpholinos are used in conjunction reflects the specific knock-down of mir. In addition, morphant phenotypes induced by mir-specific morpholinos are rescued by overexpression of the full-length Mir. In situ hybridization analysis with mesodermal- and neural-specific markers shows that morphants exhibit a delay in cell movements associated with gastrulation, epiboly, convergence, and extension. A yeast two-hybrid analysis was performed to identify binding partners that may participate with Mir during gastrulation, and Annexin V, a calcium channel protein, was isolated. At early developmental stages, annexin V transcripts colocalize with mir, but after gastrulation, annexin V mRNA becomes localized to the distal tail region and an area in the olfactory placode. At the protein level, Mir colocalizes with Annexin V when expressed in COS cells. Together, these results indicate that Mir is essential for embryonic development and that its role in early embryonic development likely involves calcium-dependent mechanisms essential during the extensive cell movements associated with gastrulation.

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Introduction

The Band 4.1 superfamily of proteins contains numerous members that provide a vital link between the plasma membrane and the cytoskeleton, thereby influencing cell growth, morphogenesis, and tumorigenesis. The FERM (Band 4.1/ezrin/radixin/moesin) domain is the hallmark of this family, allowing members of the Band 4.1 superfamily to interact with CD44, ICAM3, glycoporphin C, and other proteins and molecules associated with the plasma membrane (reviewed in Chishti et al., 1998; Nunomura et al., 1997, 2000; Serrador et al., 1997; Hemming et al., 1995). The FERM domain is usually found at the N terminus of the polypeptide, but it is located centrally or at the C terminus in more distantly related members like PTP-BAS and unconventional Myosins VII and X (Weil et al., 1996; Berg et al., 2000; Saras et al., 1994). In some members, the FERM

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Fig. 1. cDNA sequence and predicted amino acids of zebrafish Mir. The unshaded box shows the hallmark Band 4.1 FERM domain at the amino terminus of the polypeptide. Sequence similar to the 14-3-3/PH2 binding sequence found in PTPH1 is enclosed in the outlined shaded box, and the RING finger domain is shaded. The consensus cysteine and histidine residues of the RING finger are in uppercase and underlined. The mir-specific morpholinos designed to "knock-down" Mir expression are denoted as M-1, beginning 5′ with the A of the initiation methionine and including 24 nucleotides in the 5′ untranslated region (UTR). The second morpholino (M-2) was targeted further upstream and tagged with fluorescein to follow its distribution in injected embryos.
domain facilitates protein–protein interactions with the cytoskeleton (Aoyama et al., 1999; den Bakker et al., 2000). The diversity of these interactions and the presence of specific domains and/or motifs have allowed investigators to categorize and divide the Band 4.1 superfamily into several subfamilies (Takeuchi et al., 1994).

The Band 4.1 subfamily contains the founder protein Band 4.1, 4.1R, or membrane skeleton protein 4.1, was first identified in erythrocytes as a protein that binds glycoporphin C and A, Band 3, CD44, and actin microfilaments (Conboy et al., 1986; Takeuchi et al., 1994; Chishiti et al., 1998). Disruption of these interactions due to mutations in Band 4.1 compromises the integrity of the membrane structure and underlying cytoskeleton, resulting in mild to severe forms of anemia (Yawata et al., 1991). These membrane–cytoskeletal interactions via a Band 4.1 bridge are modulated by protein kinase C, cAMP-dependent protein kinase-induced phosphorylation, and calcium–calmodulin interactions (Kelly et al., 1991; Ling et al., 1988; Nunomura et al., 2000). These same modulators likely regulate other isoforms of Band 4.1, thereby ensuring that the structural integrity of the plasma membrane is maintained should Band 4.1R expression be disrupted (Yawata et al., 1997). In addition to its known structural role, Band 4.1 isoforms are also involved in recruiting the AMPA receptor subunit GluR1 to the plasma membrane (Shen et al., 2000). Receptor recruitment may be fundamentally linked to the Band 4.1 subfamily since the other subfamilies, including NBL4, ERM, and PTPH1, have mutually exclusive roles in other cells and tissues. The role(s) of the proteins in these other subfamilies are as diverse as their tissue distribution.

The NBL4 (novel Band 4.1-like protein 4) subfamily is composed of NBL4, NBL5, and Ehm2. The amino acid sequence of NBL4 is highly conserved between zebrafish, mouse, and humans (Kelly and Reversade, 1997; Takeuchi et al., 1994; Ishiguro et al., 2000). NBL4 is regulated by β-catenin, and its accumulation in human cancer cells is likely involved in the determination of cell polarity or proliferation (Ishiguro et al., 2000). Ehm2 is highly expressed in specific cancers, and like NBL4 and -5, its function is unknown (Shimizu et al., 2000). Other NBL-related proteins, including NBL6, are cataloged with the ERM, or Ezrin, Radixin, Moeisin, Merlin/Shwannomin, subfamily. Attention has focused on this subfamily since the identification of Merlin, a tumor suppressor gene mutated in human disease neurofibromatosis type 2 (Rouleau et al., 1993). The exact role of the protein product of the Merlin gene is not fully understood, but evidence suggests that it, like NBL4 and Ehm2, encodes a modulator of cell growth (Lutcharman and Rouleau, 1995).

The ERM-dependent regulation and maintenance of cell growth, motility, and shape is likely due to the actin-binding domain near the C terminus of the ERM polypeptides (reviewed in Tsukita and Yonemura, 1997, 1999; Lamb et al., 1997; Gutmann et al., 1999; Stokowski et al., 2000). In addition to their ability to interact with F-actin, ERM proteins bind to each other and to other subfamily members as homo- and heterodimers, respectively. These interactions are regulated by kinases, and the extent of phosphorylation influences the subsequent localization of the proteins to the apical plasma membrane (Krieg and Hunter 1992; Bretscher, 1989; Shaw et al., 1998; Fukata et al., 1998).

Overexpressing ERM proteins impairs cell motility, adhesion, and spreading (Lutcharman and Rouleau, 1995; Gutmann et al., 1998), possibly through the mislocalization of other proteins, including Na+/H+ exchanger 1 and ERM-binding phosphoprotein-50 (Denker et al., 2000; Gutmann et al., 1999). This information supports the notion that the correct positioning of ERM proteins, and likely other members of the Band 4.1 superfamily, is essential to transduce mitogenic signals from the plasma membrane to the cytoskeleton and vice versa.

Correct positioning of any scaffolding protein is essential for regulating cell growth and motility, but evidence also exists to indicate that members of the Band 4.1 superfamily signal by enzymatic means. In the case of the PTPH1 subfamily, members possess protein tyrosine phosphatase activity implicated in the regulation of cell growth. Ectopic expression of some PTPH1-related proteins retards cell growth and decreases cell spreading and adhesion (Ogata et al., 1999). This influence on growth and motility is attributed to the dephosphorylation of VCP (Vasolin-Containing Protein/p97/CDC48), an ATPase required for cell cycle regulation (Zhang et al., 1999; Patel and Latterich, 1998).

Since embryonic development relies on extensive cell movements, especially during gastrulation, one might predict that members of the PTPH1 subfamily are involved in regulating cell growth and spreading during embryogenesis.

To address whether members of the PTPH1 subfamily play a role in embryonic development, a zebrafish adult cDNA library was screened with a PTPH1-specific probe and a cDNA was identified that is homologous to the human gene encoding MIR (myosin regulatory light chain interacting protein; Olsson et al., 1999). Both zebrafish Mir and human MIR possess the N-terminal FERM domain, most similar to that present in PTPH1 and PTP-MEG, and a
C-terminal RING finger domain present in inhibitors of apoptosis proteins (IAP). In situ hybridization and RT-PCR analyses indicate that zebrafish mir transcripts are maternally derived and ubiquitously expressed throughout development. Overexpressing a mir mRNA encoding the full-length Mir polypeptide has no apparent effect on development, whereas the loss or reduction of protein expression using a morpholino-based knock-down strategy showed that Mir is an essential protein. The Mir morphants show trunk and tail defects arising from perturbed epiboly, convergence, and extension movements prior to and during gastrulation. Several possibilities exist to explain the morphant phenotype. Human MIR was first identified as a protein that binds the myosin regulatory light chain, and since myosin is a key component of the cortical cytoskeleton and has diverse roles including muscle contraction, cell migration and cell–cell and cell–substrate adhesion, cytoskeleton, and vesicle transport, it is likely that one or more of these processes are perturbed by the loss of Mir in zebrafish embryos. The link between MIR and myosin is compelling, but individual members of the Band 4.1 superfamily are capable of binding multiple proteins. Using a yeast two-hybrid screen to identify zebrafish Mir-interacting proteins, we found that Mir interacts with Annexin V, a protein that plays a key role in a number of Ca^{2+}-dependent processes, including anti-coagulation events, the formation of voltage-dependent calcium channels, and the inhibition of protein kinase C signaling pathways (Funakoshi et al., 1987; Demange et al., 1994; Dubois et al., 1998). This ability to bind Annexin V suggests that Mir, in addition to its possible roles in myosin-reliant events, participates in Ca^{2+}-dependent processes, and vesicle transport, it is likely that one or more of these processes are perturbed by the loss of Mir in zebrafish embryos. The link between MIR and myosin is compelling, but individual members of the Band 4.1 superfamily are capable of binding multiple proteins. Using a yeast two-hybrid screen to identify zebrafish Mir-interacting proteins, we found that Mir interacts with Annexin V, a protein that plays a key role in a number of Ca^{2+}-dependent processes, including anti-coagulation events, the formation of voltage-dependent calcium channels, and the inhibition of protein kinase C signaling pathways (Funakoshi et al., 1987; Demange et al., 1994; Dubois et al., 1998). This ability to bind Annexin V suggests that Mir, in addition to its possible roles(s) in myosin-reliant events, participates in Ca^{2+}-dependent processes that are necessary for gastrulation in zebrafish.

Materials and methods

Animals

Danio rerio adults (Superpets, London ON) were housed in dechlorinated tap water at 28°C and kept on a 12-h light/dark cycle. Embryos were collected 15 min after the beginning of a light cycle, rinsed in Instant Ocean (Aquarium Systems), and incubated at 29°C. Animals were maintained under the guidelines of the Canadian Council of Animal Care.

Isolation and identification of mir

Oligodeoxynucleotide primers (sense 5′-ATCTCCCAG-CAAGATGGACA-3′, antisense 5′-GGAACAAGCCCAA-GAGCTCAG-3′) corresponding to an EST sequence reported for the partial-length zebrafish PTPH1 cDNA (Gong et al., 1997) were added to a PCR mixture containing 1 μg of adult zebrafish cDNA and 2.5 U of AmpitGold (Perkin-Elmer). The reaction proceeded for 30 cycles of 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C. After amplification, an aliquot of the PCR mixture was analyzed on an agarose gel containing ethidium bromide to confirm the presence of a single amplicon of expected size. Once confirmed, an aliquot of the reaction was ligated into pT-Advantage (Clontech). Recombinant clones were restriction enzyme-digested, analyzed on an agarose gel, then sequenced and compared with the 182-bp PTPH1 cDNA reported by Gong et al. (1997). The 182-bp fragment was gel purified. 32P-labeled (Ambion), and used as a probe to screen approximately 1 × 10^6 clones from an adult zebrafish λ-Zap cDNA library (generously provided by Dr. D. Grunwald, University of Utah). Twenty positive plaques were identified, excised to yield phagemids, then restriction enzyme-digested and analyzed on agarose gels to determine the size of the inserts. Two clones (29 and 34.1) were selected for subsequent analysis, and one (34.1) was sequenced on both strands. Homology searches were done by using the BLAST server available at the NCBI GenBank database.

Mapping mir

Zebrafish mir was mapped by using the LN54 zebrafish–mouse radiation hybrid panel (Hukriede et al., 1999). Primers used in the reaction were antisense (5′-TGGCA-GAGCATTTTGAGAGG-3′) and sense (5′-AAAGTGAGCCAGGGCTGTAGTGAGG-3′). Aliquots from the PCR were electrophoresed on agarose gels, and lanes were scored for the presence of an ampiclon. Results were collected and analyzed by using RHMAPPER (http://mgchd1.nichd.nih.gov:8000/zfrh/beta.cgi after Hukriede et al., 1999).

Isolation of mouse Mir

Overlapping expressed sequence tags (ESTs) for mouse sequence, similar to human MIR, were assembled, and oligodeoxynucleotide primers were designed to isolate sequence encoding the full-length mouse Mir cDNA. A day 7 mouse embryo cDNA library (Clontech) was used as template in the PCR. The amplicon generated from the reaction was ligated into pT-Advantage and sequenced to confirm its identity to human MIR.

In situ hybridization

Embryos were collected from several developmental stages (2–4 cell, 50% epiboly, 1 somite, 10–14 somite, 18 somite, and prim 6), and prepared for in situ hybridization using the protocol outlined by Jowett (1999). Embryos were incubated in hybridization solution containing digoxigenin (DIG) probes (sense or antisense) generated by using the Maxiscript kit (Ambion). Embryos were washed extensively to remove unbound probe, blocked in 2 mg/ml bovine serum albumin, 5% goat serum, 5% dimethyl sulfoxide, and
Fig. 3. *In situ* hybridization showing the expression pattern of zebrafish *mir* mRNA during embryogenesis. (A) A control embryo hybridized with the sense strand, DIG-labeled probe shows no NBT-BCIP-positive signal. (B) Dorsal view showing *mir* signals in blastomeres of the 4 cell-stage embryo. (C) Expression is continuous throughout development and is ubiquitous in all cells seen here in a lateral view of an embryo at approximately 10 h postfertilization. This trend in expression pattern is maintained in embryos throughout somitogenesis (D). Higher magnification images of the head reveal ubiquitous expression in the head of 18 somite and 26 somite embryos (E, F). Decreasing the time in the chromogenic reaction accounted for the apparent reduction in *mir* staining in the embryo shown in (F).
PBS–Tween 20 then incubated overnight at 4°C in blocking solution containing anti-DIG antibody conjugated to alkaline phosphatase (Roche Diagnostics). Following an extensive wash in PBS and three rinses in NTMT (100 mM NaCl, 100 mM Tris–HCl, pH 9.5, 50 mM MgCl₂, and 0.1% Tween 20), embryos were incubated in NTMT containing NBT/BCIP (4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate). Following the color reaction, embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), transferred to methanol, then cleared in benzylbenzoate/benzyl alcohol prior to examination using differential interference contrast microscopy. Photomicrographs were taken by using a Leica Wild MPS52 camera mounted on a Leica DMRBE microscope, and digital images were captured with a Hamamatsu digital camera. In situ hybridization analysis was also done with the annexin V probe and with probes to identify rhombomere 3 and 5 (krox20), notochord (ntl), somites and axial tissue (myoD), prechordal mesoderm (hhg-1), and neurectoderm (dlx-3).

Reverse-transcriptase PCR

Stage-specific RNA was isolated from embryos at the 64-cell, sphere, shield, early somitogenesis, and 14-somite stages, and converted into first strand cDNA using Superscript reverse transcriptase (Invitrogen). The cDNA was used in a PCR (as described above) with primers for mir (sense 5'-CGAGCCGCCAGTGACTTTACCGC-3', antisense 5'-GTCGTACACTTCCTTAGATGTGCG-3'), anxV (sense 5'-CGCGATCCAATGCATCATCACC-3', antisense 5'-CATTTAGGAGAGCGAGTAGGC-3') or for the constitutively expressed ribosomal gene L37A (sense 5'-CCAAGCCCAAGAAG-3', antisense 5'-GTCCTACGCTCCTTCAGTC-3'). Results were analyzed on a 1% agarose gel containing ethidium bromide.

Yeast two-hybrid screen

A yeast two-hybrid screen was performed by using a day 7 mouse embryo cDNA library (Clontech). cDNAs encoding the complete open reading frame of zebrafish Mir, as well as deletions of the N or C terminus, were fused to the GAL4 DNA binding domain in the pAS2.1 bait vector. DNA was transformed into the yeast strain AH109 according to manufacturer’s instructions, then transformed with a mouse embryo cDNA library fused to the GAL4 activating domain contained in pGAD10 (generously provided by Dr. J Verdi, Maine Medical Research Institute). Approximately 1 × 10⁶ transformants were screened for activation of the his-3 reporter gene on medium lacking histidine, tryptophan, and leucine and containing 5 mM 3-aminotriazole. Colonies appearing within 7 days were screened for β-galactosidase activity by using a filter lift assay. Positive clones were isolated from yeast and transformed into Esch-
erichia coli. Clones were also transformed into yeast containing the zebrafish Mir bait construct to confirm the positive interaction.

Isolation of the zebrafish annexin V homolog

Overlapping ESTs for zebrafish sequence similar to chick Annexin V (AI384320, BE201296, BE557472) or Oryzias latipes (killifish) max2 (AW128143, AW184217) were assembled and oligodeoxynucleotide primers designed to isolate sequence encoding a full-length zebrafish annexin V cDNA (see Fig. 6). Zebrafish first strand cDNA (oblong stage, 3.5 h postfertilization) was used as template in the PCR. The amplicon generated from the reaction was ligated into pT-Advantage and sequenced to confirm its identity to rat Annexin V. Following isolation and sequencing, zebrafish annexin V was subcloned and used in a direct yeast two-hybrid screen with mir.

Zebrafish Mir and Annexin V coexpression in COS-7 cells

The cDNA encoding the complete zebrafish Mir protein or ones containing various deletions in the Mir coding region were cloned into the pEGFP-C2 vector (Clontech) to make enhanced green fluorescent protein expression constructs. Vectors were transfected into COS-7 cells by using a CaCl\textsubscript{2} method. After 16 h of incubation, cells were rinsed with 1× PBS and grown at 37°C with 5% CO\textsubscript{2} in DMEM media containing 10% fetal bovine serum and 1% penicillin/streptomycin. Visualization of the EGFP fusion proteins was done with a Leitz Orthoplan microscope equipped with the PloemPak epifluorescence system. Digital images were captured with a Sony XC-75 digital camera modified for image integration (Empix Imaging, Mississauga, ON). Vectors encoding zebrafish Annexin V fused to dsRed were transfected into COS-7 cells and the expression of the fusion proteins was followed using the protocol described above. For coexpression analysis, plasmids containing pEGFP-Mir and dsRed-Annexin V were transfected into COS-7 and the expression of the fusion proteins examined using a Zeiss laser scanning confocal microscope. Transfection with a plasmid encoding either the Mir or Annexin V fusion protein, with one encoding only the fluorescent protein, served as controls.

Morpholino antisense knock-down strategy

Antisense oligodeoxynucleotide morpholinos (M-1 5′-TTTGGAGGAGGTTTTGTTGG-3′ and M-2 5′-CCAGTCTCTGTACGAAAAACACTAC-3′) (Gene Tools, Corvallis, OR) were designed to complement the 5′ untranslated region (UTR) sequence upstream of the AUG site of mir (M-1 and M-2, Fig. 1). A control morpholino, complementary to human β-globin (CCTTATACCTCAGTTAACAATTATA), was also used to determine whether the effects seen were Mir-specific. Antisense oligonucleotides were suspended in Danieau solution [58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO\textsubscript{4}, 0.6 mM Ca(NO\textsubscript{3})\textsubscript{2}, 5.0 mM Hepes, pH 7.6], then microinjected, separately and/or in combination, into 1–4 cell-stage zebrafish embryos. The β-globin control morpholino was also resuspended in Danieau and injected into 1–4 cell-stage embryos. Each embryo was injected with approximately 1 nl of solution at a needle concentration ranging from 0.5 to 10 mg/ml. Embryos were assayed for visible developmental defects throughout development, and those exhibiting abnormal phenotypes (morphants) were fixed and processed for in situ hybridization by using neural- and mesodermal-specific probes. To test whether the morpholinos were specifically knocking down the levels of Mir, the morphants should be rescued by supplementing them with exogenous Mir. Embryos were first injected with pEGFP-Mir; a plasmid containing sequence encoding full-length Mir or the empty vector alone (pEGFP), to ensure that Mir overexpression or the plasmid by itself would not perturb development. Experiments were repeated and 1 nl of pEGFP-Mir (0.025 mg/ml) was co-injected with 1 nl of morpholino (5 mg/ml). Embryos were observed for 24 h and phenotypes compared with controls at the same hour postfertilization (hpf).

Detection of apoptosis

In situ hybridization analysis of morphants suggested that apoptosis might contribute to the gastrulation defects seen in Mir-depleted embryos. Embryos injected with the M2 morpholino or un.injected controls were analyzed for apoptosis by using the TACS XL-DAB Detection Kit (Trevigen). Briefly, embryos from several stages were collected, dechorionated, and fixed in 4% PFA in PBS, then quenched and labeled as per manufacturer’s instructions. TdT-labeled embryos were incubated for 30 min at 37°C in a solution containing anti-BrdU antibody, then washed in PBS and incubated in Strep-HRP solution. Embryos were placed in diaminobenzidine (DAB) solution (1/5000 dilution of DAB in PBS and 0.3% hydrogen peroxide) for 2–7 min, then fixed in 4% PFA in PBS and visualized as described earlier. For a positive control, embryos were treated with TACS-nuclease during the incubation with labeling solution to fragment DNA, then treated as described above.

Results

Isolation and identification of zebrafish mir

A cDNA encoding a region of zebrafish PTPH1 was used to construct primers to make a probe to screen an adult zebrafish cDNA library. The primary screen yielded 20 positive plaques, 5 of which were confirmed in a second screen. These 5 cDNAs were restriction mapped and found to be identical based on fragment size. Since all 5 were likely the same cDNA, 1 (p34.1) was sequenced on both
strands. Overall, p34.1 is 2589 nucleotides in length and the largest open reading frame of 1419 nucleotides codes for 473 amino acids (Fig. 1). Homology searches through GenBank revealed that p34.1 does not encode PTPH1 as expected, but instead encodes a novel Band 4.1 superfamily member related to a human protein, MIR (Olsson et al., 1999), and the Drosophila gene product CG12489 (Fig. 2A). Although BLAST searches through the GenBank databases would suggest that we had cloned the zebrafish homolog of the human Mir cDNA, we tested this further by mapping the p34.1 cDNA to the zebrafish–mouse LNS54 radiation hybrid panel previously described by Hukriede et al. (1999). The p34.1 cDNA maps to zebrafish linkage group 19 within 4.81 cR from csk2b, and a logarithm of the likelihood ratio for linkage (Lod) score of 12.9. Other genes near csk2b include rxre and ntl. The human homologs around mir were identified from BLAST searches and the chromosome location of the human genes identified through the online version of the Mendelian Inheritance of Man (www.hgmp.mrc.ac.uk/omim/searchomin.html). Human CSK2b maps to 6p21.3, RXRE to 6p21.3, NTL to 6q27, and MIR to 6p23–p22.3. The zebrafish clone p34.1 is 81% and 71% identical to human MIR at the nucleotide and amino acid levels, respectively. Based on this homology, the fact that p34.1 maps to approximately 570 kb of csk2b and that human MIR and CSK2b are located on the same chromosome, we have designated the p34.1 cDNA as the zebrafish homolog mir and the polypeptide predicted from the sequence as Mir, to fit the convention of naming genes and proteins in zebrafish.

Mir contains the hallmark FERM domain (outlined in Fig. 1) common to all Band 4.1 superfamily members. The N-terminal region of Mir is similar to phosphatase-containing members of the Band 4.1 superfamily, namely PTPH1 and PTP-MEG. The C terminus of Mir, however, differs significantly from the two PTPH1 subfamily members, as it encodes a cysteine-rich zinc finger (shaded region, Fig. 1), similar to that in mouse and human inhibitor of apoptosis proteins (IAPs) (Fig. 2B). Although there is no recognizable protein tyrosine phosphatase domain, Mir contains a putative 14-3-3 binding site (RSRSRE, boxed-in shaded region, Fig. 1), similar to the consensus-sequence reported by Fu et al. (2000). Interestingly, this consensus site does not exist in human MIR or mouse Mir, but is present in PTPH1 (Zhang et al., 1997). Several putative sites for regulation by calcium/calmodulin-dependent protein kinase II, protein kinase CK II, cAMP-dependent protein kinase A, and protein kinase C, were identified in Mir (PhosphoBase: www.cbs.dtu.dk/databases/PhosphoBase), but whether or not any or all are used by a kinase in vivo was not determined.

Zebrafish mir expression in embryogenesis

Although mir was isolated from an adult cDNA library, we anticipated finding embryonic expression since the probe used in the initial screen was generated from first strand embryonic cDNA. In situ hybridization was used to confirm that mir is expressed during embryogenesis. Embryos hybridized to antisense, DIG-labeled mRNA probes revealed that mir is expressed at the 4 cell stage (Fig. 3B). The expression before the midblastula transition indicates that mir transcripts are maternally derived (Kane and Kimmel, 1993). Expression continues throughout development, and at approximately 10 h postfertilization, transcripts are ubiquitously expressed in all cells of the early somite stage embryo (Fig. 3C). This ubiquitous expression is maintained through late embryogenesis (Fig. 3D–F), and presumably into the adult, as an adult library was used in the initial screen to isolate mir. Embryos hybridized with the sense strand control probe showed no detectable signal (Fig. 3A). RT-PCR was used as an independent approach to analyze the expression of mir during embryogenesis. Primers to the constitutively expressed ribosomal subunit L37A were first used to confirm the presence of first strand cDNA reverse transcribed from mRNA isolated out of pre- and post-MBT stage embryos. An amplicon of the expected size (247 bp) indicates that first strand cDNA was present in all stages tested and therefore available as template to the mir primers (Fig. 4A). In another PCR, using mir-specific primers, results clearly show that mir is expressed prior to the midblastula transition and later during gastrulation, early and midsomitogenesis (Fig. 4B).

Zebrafish Mir-interacting proteins

Members of the Band 4.1 superfamily interact with various membrane- and cytoskeletal-associated components, integral membrane and cytosolic proteins, and F-actin. Although human MIR was first identified as a protein that binds to the regulatory light chain of myosin (Olsson et al., 1999), there is precedence for a single member of the Band 4.1 superfamily to have multiple binding partners (Algrain et al., 1993; Shen et al., 2000). To address this, a yeast two-hybrid screen was used to identify proteins that recognize zebrafish Mir (Fig. 5A). A mouse embryonic (gastrula-stage) cDNA library, originally selected because of its large number of primary clones, was used as the prey together with a cDNA encoding the full-length Mir as bait. After screening approximately $1 \times 10^6$ transformants in a sequential transformation strategy, 50 colonies grew on triple dropout media containing 5 mM 3AT. Colonies were tested for the expression of β-galactosidase and 4 positives were isolated for further investigation. Sequence analysis revealed that 3 cDNAs encoded out-of-frame or nonsense polypeptides, but the fourth was in frame and encodes the first 196 amino acids of mouse Annexin V. Since the complete Annexin V protein is 319 amino acids in length (Rodriguez-Garcia et al., 1996), the Mir binding region must lie somewhere within 60% of the complete polypeptide. The interaction was tested further and confirmed by transfecting yeast with constructs encoding zebrafish Mir and the amino terminus of mouse Annexin V. Since the two-hybrid anal-
(A) Schematic representation of the expression of different constructs. (B) Table showing interactions of various constructs. (C) Fluorescence images showing the expression and localization of different constructs over time.
Fig. 6. cDNA sequence and predicted amino acids of zebrafish Annexin V. (A) Overlap of two zebrafish ESTs (AW128143 and AW184217) predicted to span the open reading frame of annexin V. (B) The cDNA sequence for annexin V was isolated by RT-PCR using embryonic first strand cDNA and oligodeoxynucleotide primers complementary to coding sequence at the amino terminus and in the 3' UTR (underlined). Amino acids predicted from the cDNA sequence are denoted by using standard three letter uppercase abbreviations.

Fig. 5. Yeast two-hybrid analysis revealed that Mir interacts with mouse and zebrafish annexin V. (A) Yeast cells containing the full-length Mir, or Mir deletion/truncation constructs, were transformed with a mouse day 7 embryonic cDNA library. Mir43 encompasses the first 43 amino acids of Mir, Mir/4.1 is missing a significant portion of the FERM domain and the putative 14-3-3 binding site in the central region of Mir, and Mir/RING lacks part of the central region, the entire RING finger, and the C terminus of the polypeptide. (B) Yeast that grew on dropout media containing 5 mM 3-aminotriazole were picked and interactions tested using a -galactosidase filter assay. A plus symbol (+) denotes yeast that grew and tested positive for the expression of -galactosidase. A minus (−) symbol denotes no growth. After the initial identification of a positive Mir interactor, mouse annexin V (AnxV), the zebrafish homolog was cloned and screens were repeated using it as prey and full-length Mir as bait. Yeast containing Mir or annexin V alone did not propagate on dropout media, and only full-length Mir was capable of binding mouse and zebrafish annexin V. (C) Representative laser scanning confocal images showing that EGFP-Mir colocalizes with ds-RED-AnnexinV in COS-7 cells. The extent of this colocalization is seen in yellow in the merged images. The particle denoted by the arrow was followed and images collected over time. The travel of this particle to the periphery of the cell and subsequent return can be seen more clearly at http://publish.uwo.ca/~gkelly/movie.htm (to be released following publication). The data are representative of multiple transfection experiments. Scale bar in (C) is 10 μm and is equal for all panels.
ysis often identifies spurious interactors, and given that the original screen was between distantly related vertebrate species, it was necessary to demonstrate that zebrafish Annexin V interacts with zebrafish Mir (Fig. 5).

The sequence corresponding to zebrafish annexin V has not been reported previously, so sequence data from zebrafish ESTs similar to annexin V or max2 (Osterloh et al., 1998) were retrieved from GenBank (Fig. 6A) and primers designed to use in a PCR strategy. Oblong stage (3.5 h postfertilization) first strand cDNA was used as template to amplify sequence encoding the open reading frame of annexin V. An amplicon of approximately 900 bp was isolated and sequenced (Fig. 6B), and BLASTX searches revealed that it was the zebrafish homolog of rat Annexin V and O. latipes max2. The polypeptide predicted from the open reading frame sequence contains 315 amino acids, 61% of which are identical to rat Annexin V. Having identified zebrafish Annexin V, the cDNA could now be used in a yeast two-hybrid assay to confirm that the protein binds Mir. Results show that yeast containing zebrafish Mir and zebrafish Annexin V survive the dropout selection, whereas those containing either Annexin V or Mir alone do not (Fig. 5B). A β-galactosidase assay confirmed the interaction between the two polypeptides (data not shown).

Having satisfied the criteria that zebrafish Mir binds both mouse and, more importantly, zebrafish Annexin V, we were next interested in identifying the Annexin V binding region within Mir. Two-hybrid screens with cDNAs encoding full-length Annexin V and Mir missing the FERM domain (MirΔ4.1), the RING finger domain (MirΔRING), or a Mir polypeptide truncated after the 43rd amino acid, prevented the propagation of yeast on dropout media (Fig. 5A). Positive controls, recommended by the manufacturer (Clontech), propagated indicating that the yeast and the assay were functioning normally. Together, these results indicate that the entire Mir polypeptide is needed for its interaction with Annexin V, whereas the C-terminal (40%) of Annexin V, as evident from the initial screen, is not necessary (Fig. 5B). The colocalization of the Mir and Annexin V was examined in COS-7 cells to validate the interaction identified in the yeast two-hybrid analysis. Expression vectors encoding EGFP-Mir and dsRed-Annexin V were constructed and then introduced into cells to follow the distribution of the proteins. The localization of Annexin V and Mir was determined indirectly by dsRed and EGFP fluorescence, respectively. Red and green fluorescence was seen in discrete, punctate regions throughout the cell (Fig. 5C), and was uniformly distributed within the cytoplasm of cells transfected with the empty vector encoding only the fluorescent protein (not shown). The fluorescence patterns of the Annexin V and Mir fusion proteins appeared similar, and when the two color channels are merged, the extent of colocalization is seen as a yellow signal (Fig. 5C). Fluorescence was also monitored in live cells to determine if the patterns would change over time. Results indicate that particles exhibiting both green (Mir) and red (Annexin V) fluorescence move rapidly throughout the cytosol (Fig 5C). Although there does not appear to be a definite pattern to the movement and the content of the particles are currently not known, particles positive for Mir and Annexin V stain positively for the early endosome marker Rab5 (M.N.K. and Kelly, G.M.K. unpublished observations).

The colocalization seen in live cells lends support to the notion that the interaction identified in our yeast two-hybrid screen is bona fide. If the interaction between Mir and Annexin V is genuine, we would expect that the mRNAs encoding these nonsecreted proteins to coexpress in the embryo. Given this hypothesis and since mir is expressed maternally (Fig. 4), we would expect to first see annexin V mRNAs in pre-MBT stages. To address this, we used RT-PCR and the annexin V-specific primers shown in Fig. 6B. Results show that annexin V transcripts, as evident by the presence of a 300-bp amplicon, are present in all stages tested (Fig. 7A). This developmental profile showing that annexin V and mir are expressed at the same time would meet one criterion that Mir interacts with Annexin V throughout embryogenesis. However, further analysis would be necessary to show that mir and annexin V are coexpressed in the same cell type. In situ hybridization using DIG-labeled, sense and antisense mRNA probes was used to determine the spatial expression patterns of zebrafish annexin V during embryogenesis (Fig. 7B–D). Embryos at early (1.25–4 h postfertilization), mid (4–12 h postfertilization), and late stages (12–35 h postfertilization) of development were used in the analysis. Signals were present in early cleavage stage embryos, but no region or specific cell type appeared to be enriched with annexin V mRNA (Fig. 7B). This global expression is maintained as cells spread over the yolk during epiboly (Fig. 7C). During somitogenesis, however, annexin V expression becomes restricted to the distal tail region and to an area in the olfactory placode, immediately adjacent to the eyes (arrows, Fig. 7D and E). There is also discrete staining in the hatching gland (single arrow, Fig. 7E). These results together with those from the RT-PCR analysis indicate that Annexin V could interact with Mir in all cells at early stages of embryogenesis, but only in very specific groups of cells later on in development. Further confirmation of an interaction at the protein level using whole-mount embryos and anti-Mir-specific antisera is in progress (M.N.K. and G.M.K. unpublished observations).

Deregulation of zebrafish Mir expression in embryos

With the various cDNAs used in the yeast two-hybrid analysis, and given the evidence that mutations in mouse Merlin and human 4.1R lead to specific perturbations in cell structure/function, we postulated that one or more Mir deletion constructs might serve as a dominant negative or positive if expressed in the intact embryo. Embryos were injected with mRNA encoding the full-length Mir or DNA constructs encoding either a Mir polypeptide truncated at
amino acid 361 in the FERM domain or one lacking the entire RING finger. Although it was unfortunate that there were no obvious developmental perturbations when full-length mir in a DNA construct was injected, this shortfall allowed us the opportunity to use the mir derived from a DNA construct to rescue morphants (described later). In contrast, embryos injected with the DNA constructs lacking the RING finger exhibited defects, including delayed gastrulation and tail defects (data not shown). Since these results suggested that the loss of the C terminus of the polypeptide, and more specifically the loss of the RING finger, blocked the normal function of Mir prior to or during gastrulation, then the loss of the entire protein would be expected, at least, to have a similar outcome. However, proteins in the Band 4.1 superfamily are known to serve in functionally redundant roles, allowing the loss of one member to be compensated functionally by a relative (Doi et al., 1999). Morpholino technology as a posttranscriptional means to disrupt Mir translation was used to address whether or not another Band 4.1 superfamily member can compensate for the loss of Mir, or if Mir is essential for development. Morpholino-containing antisense oligonucleotides, specific to the zebrafish mir sequence in the 5’ UTR of the message (M-2, Fig. 1), or immediately at the AUG initiation methionine and spanning a stretch upstream of the 5’ UTR (M-1, Fig. 1), were microinjected into zebrafish embryos to determine what effect reduced Mir expression has on development (Table 1). The control β-globin morpholino has little effect on mortality rate and only a slight effect on gastrulation (1–3% incomplete gastrulation) and subsequent trunk and tail development (2–10% tail defects) when embryos were injected with as much as 10 ng (Table 2). The M-2 morpholino was fluorescein-tagged, thereby allowing it to be followed in a live embryo throughout development and ensuring even partitioning of the morpholino among cells (Fig. 8). Fluorescent (Fig. 8B, D, F, H, and J) and complementary differential interference contrast images (Fig. 8A, C, E, G, and I) reveals that the morpholino is distributed to all cells. While embryogenesis prior to gastrulation appears unaffected by the presence of the morpholino, developmental perturbations are seen during gastrulation (6–10 hpf) and somitogenesis (10–24 hpf) (Fig. 8G). This includes a general delay in gastrulation events resulting in trunk and tail defects such as the failure of the embryos to extend along the anteroposterior axis when compared to uninjected or Danieu-injected siblings at the same time hpf (Fig. 8G and I). Similar defects were observed upon expression of the Mir lacking the RING finger (not shown).

As noted above, early development in Mir-depleted embryos appeared normal. However, by gastrulation, these embryos were developing noticeably slower than their uninjected or Danieu-injected siblings. By 12 hpf when uninjected embryos had completed gastrulation and begun somitogenesis, 2–29% (depending on dose and morpholino) of the embryos injected with the mir-morpholino had only achieved 75% epiboly (Table 1, see Fig. 10A and B). Severely affected embryos failed to complete epiboly and eventually died. The delay in epiboly, or in some cases its complete absence, resulted in increased mortality as well as altered trunk and tail structures (Table 1). While the two mir-specific morpholinos retarded epiboly, leading to trunk and tail defects, they did so at different rates. In general, M-1 was more potent than M-2, and at the highest dose tested (10 ng), it induced 69% mortality compared with 55% caused by M-2. Results are the direct result of the morpholino as unfertilized embryos were removed shortly after microinjection and are not included in reported mortality rates. The lowest amount injected (0.5 ng) resulted in only 37% of the embryos dying or developing trunk and tail defects. The increased mortality compared with Danieu-injected embryos can be attributed to defects that occur during and following gastrulation since few fertilized embryos died prior to gastrulation. As a further test of the efficacy and specificity of the mir-specific morpholinos, we coinjected 0.5 ng of each morpholino and recorded the number of morphants that developed. This amount was chosen since our previous results had shown that individual morpholinos at that concentration had little effect on either mortality or gastrulation. Coinjection of the morpholinos resulted in 43% mortality, with 34% of the surviving embryos exhibiting trunk and tail defects. The synergistic effect of coinjecting the morpholinos resulted in greater mortality and gastrulation defects than that induced by 0.5 or 1.0 ng of either morpholino alone. We therefore attribute these results to the specificity of each morpholino to disrupt Mir levels.

The specificity of the morpholinos was also confirmed by rescuing the Mir-depleted morphants with exogenous full-length Mir expressed from a CMV-driven plasmid. As previously described, injecting pEGFP-Mir or the empty vector pEGFP has no effect on embryogenesis. Embryos coinjected with the M2 morpholino (5 ng) and 25 pg of pEGFP-Mir displayed reduced mortality, as well as reduced gastrulation and tail defects relative to M2 alone (Table 3). At similar concentrations, the rescue of M1-induced morphants using the Mir plasmid was not as successful as that for M2, possibly owing to M1’s ability to act in a more potent manner as described earlier. Nevertheless, the ability of exogenous Mir to rescue M1 or M2 morphants reflects the specificity of the morpholinos in depleting the endogenous levels of Mir in the embryo. These results indicate that Mir is required for zebrafish embryogenesis, but it still does not address how Mir functions in embryos.

The presence of the RING finger in zebrafish Mir, and as described earlier its similarity to that in inhibitor of apoptosis proteins (IAPs), raises the possibility that Mir depletion induces apoptosis during embryogenesis. A TUNEL assay for apoptosis was used to test the notion that the trunk and tail defects in Mir-depleted embryos are associated with the death of cells at or during epiboly, leading to delayed gastrulation. M1- and M2-injected embryos were processed for the TUNEL assay and the results compared to positive
and negative controls (Fig. 9). In the positive control, nuclease treatment causes fragmentation and a signal to indicate BrdU incorporation and apoptosis is seen as a dark brown spot in the blastomere nuclei of a pre-MBT stage embryo (Fig. 9A). The staining in uninjected negative control and a morpholino-injected embryo prior to MTB is uniform and there are no signs of apoptosis (Fig. 9B and C, respectively). The numbers of cells undergoing apoptosis are easily seen in the animal pole view of a nuclease-treated embryo at the onset of gastrulation (Fig. 9D). The embryo was photographed so the plane of focus would reveal the leading edge of the cells undergoing epiboly toward the vegetal pole (arrows, Fig. 9D). During epiboly, there were no obvious cells undergoing apoptosis seen in either uninjected embryos (Fig. 9E) or morpholino-injected embryos (Fig. 9F). A few apoptotic cells were seen in the central nervous system of morpholino-injected embryos at the midto-late somite stages, but their numbers were comparable to that in uninjected control embryos examined at similar stages (data not shown). The failure of morpholino-injected embryos to incorporate BrdU above levels seen in uninjected control embryos indicates that the Mir-depletion does not result in apoptosis leading to delayed gastrulation.

Since apoptosis was eliminated as a contributor to the delay in gastrulation seen in morphants, then other factors must be responsible for the trunk and tail defects that are the common readout arising from the depletion of Mir. In severe cases, morphants fail to undergo or complete gastrulation. This abrupt cessation to development would suggest that cell movements have been perturbed either immediately before and/or during gastrulation. To test this hypothesis, embryos were injected with a morpholino or Danieau solution then monitored visually throughout the first 24 h of development. Results show that, while some morphants finish gastrulating, they do so more slowly than controls at the same hpf (Fig. 10A and B). In the time it takes control embryos to complete gastrulation, cells in morphants had only migrated 75–80% over the yolk (arrows, Fig. 10B).

Fig. 7. RT-PCR and in situ hybridization analyses showing the temporal and spatial expression patterns of zebrafish annexin V mRNA during embryogenesis. (A) Reverse-transcriptase PCR of embryonic cDNA reveals that annexin V is expressed throughout development. First strand cDNA was synthesized from four different embryonic stages (1, pre-MBT; 2, shield; 3, early somitogenesis; and 4, midsomitogenesis), and a 300-bp fragment was amplified with primers specific for zebrafish annexin V. Lanes designated plus (+/H11001) or minus (−/H11002) refer to the presence or absence of reverse transcriptase in the first strand reaction. (B) In situ hybridization shows that maternal annexin V transcripts are present at the 16-cell stage. (C) At later stages and following the midblastula transition, annexin V is expressed ubiquitously in all cells, as seen in this lateral view of an embryo at approximately 50% epiboly. The darker staining regions along the periphery are edge effects and do not represent regions enriched with annexin V transcripts. (D) During somitogenesis, the annexin V signal becomes localized to the olfactory placodes, hatching gland, and where the tail joins the yolk (arrow). (E) Head-on view of an embryo at midsegmentation showing the intense labeling of annexin V in the areas of the olfactory placodes (twin arrows), and in the hatching gland (single arrow).
This delay, in morphants that complete epiboly, translates into defects arising in the trunk and tail. Imperfections in the somites are obvious (arrows, Fig. 10D), and their overall width and shape, as seen in a morpholino-injected embryo (Fig. 10D), are considerably different from those in a control, uninjected or Danieau-injected embryo, 16 hpf (Fig. 10C). Later staged morphants are shortened along the anterior–posterior axis and also have numerous defects when compared with controls at 24 hpf (Fig. 10E and F). Tail defects range from minor to severe curvatures of the tail. Examination of embryos during early somitogenesis reveals no disruption of Kupffer’s vesicle, but there are defects in yolk plug closure (data not shown). Other morphant phenotypes include elongated, kidney bean-shaped embryo bodies (not shown), suggestive of altered cellular movements associated with gastrulation, such as epiboly, convergence, and extension.

To investigate whether the delay in gastrulation leading to irregular notochord and somite formation is accompanied by perturbations in the expression of specific neural or mesodermal markers, control embryos and Mir morphants were fixed at times during the segmentation period (14 h postfertilization) and processed for in situ hybridization (Fig. 11). Krox20, a neural-specific marker, is normally expressed over the dorsal midline in rhombomeres 3 and 5 in uninjected or Danieau-injected controls (Fig. 11A). The krox20 pattern in Mir morphants differs significantly from controls, appearing as two closed doublets on either side of the embryonic midline (Fig. 11B). The embryo proper develops in the morphants, albeit more slowly than that in controls at the same hpf. The expression of the pan-mesodermal marker nt1 indicates that the notochord is present in mild and severe morphants, but it often appears crooked or twisted (Fig. 11D). In severe morphants, which fail to close the yolk plug, nt1 staining is broadened mesolaterally and compressed along the anteroposterior axis (inset, Fig. 11D). In addition, the myoD marker reveals abnormal paraxial mesoderm staining in Mir morphants, as somites appear broader and more irregular, especially in the posterior (Fig. 11F) relative to controls (Fig. 11E). Again, in severe morphants, the extent of myoD staining in somites is broadened mesolaterally and compressed along the anteroposterior axis (inset, Fig. 11E). This abnormal pattern of myoD expression translates later into the disorganized array of somites flanking the notochord. It is interesting to note that, although not all embryos had identical staining patterns, the tissue disorganization resulting from the morpholino injection was consistent. To examine the differences in how depleting Mir affects cells development in anterior versus posterior tissues, embryos were microinjected with mir morpholino then double stained for hgg-1 and dlx-3 (Fig. 11G and H). In both control and morphant embryos, hgg-1 is expressed in the prechordal plate and appears as an arrowhead-shaped band at the anterior region of the embryo. The pattern of dlx-3, normally expressed in anterior and posterior neuroectoderm, appears similar in the anterior region of control and mor-

Fig. 8. Mir morpholino-injected embryo showing the distribution of the fluorescein-tagged, antisense M-2 oligo through early-to-mid embryogenesis. (A, C, E, G, and I) Differential interference contrast micrographs corresponding to complementary images of the same embryo examined by fluorescence microscopy (B, D, F, H, J). (A–F) The morpholino has no obvious effect on early developmental stages (0–12 hpf). (G, H) Trunk and tail defects include a shortened tail and misshapen somites following the completion of epiboly and gastrulation. An uninjected embryo (I), displays no autofluorescence (J).
pholino embryos. In the posterior domain by the otic vesicles, however, dlx-3 staining covers a wider area in the morpholino-injected embryos. While Mir depletion generally disrupts epiboly, its effect on convergence and extension is restricted to posterior neural and mesodermal tissues that results in abnormal trunk and tail development.

Discussion

The Band 4.1 superfamily of proteins contains members that provide structural integrity to the cell and ones implicated in signal transduction and regulation of cell growth. Based on structure/function analysis, it appears that these proteins are ideally suited to serve many functions, possibly in combinatorial roles. In addition, members of at least one subfamily are unique since they possess a catalytic domain in combinatorial roles. In addition, members of at least one subfamily are unique since they possess a catalytic domain. That it is expressed maternally, well before the birth of neurons, is evidence for a role other than in nerve cell motility as reported by Olsson et al. (1999, 2000). Further-more, our analysis shows that Mir and its human homolog MIR, identified in a yeast two-hybrid screen, is expressed preferentially in neurons in the hippocampus and cerebellum (Olsson et al., 1999; 2000). Sequence analysis and mapping confirm that we have identified the zebrafish homolog of human MIR.

The domains identified in zebrafish Mir suggest, but by no means prove, that Mir interacts with other proteins, both in embryonic development and later in differentiated adult cells. That it is expressed maternally, well before the birth of neurons, is evidence for a role other than in nerve cell motility as reported by Olsson et al. (1999, 2000). Furthermore, our analysis shows that Mir and its human homolog MIR are more closely related to the PTPH1 subfamily rather than the ERM subfamily as reported by Olsson et al. (1999).

Table 1

Effects of morpholino “knock-down” on zebrafish embryogenesis

<table>
<thead>
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<th></th>
<th>Danieau 0.5 µg/µl*</th>
<th>0.5 µg/µl</th>
<th>1.0 µg/µl</th>
<th>5.0 µg/µl</th>
<th>10 µg/µl</th>
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<tr>
<td></td>
<td>M-1</td>
<td>M-2</td>
<td>M-1</td>
<td>M-2</td>
<td>M-1</td>
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<tr>
<td>Dead</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>19 (7.3%)</td>
<td>101 (22%)</td>
<td>233 (13%)</td>
<td>303 (43%)</td>
<td>140 (32%)</td>
</tr>
<tr>
<td>Gas. Failureb</td>
<td>1</td>
<td>15</td>
<td>15</td>
<td>45</td>
<td>12</td>
</tr>
<tr>
<td>Trunk &amp; Tail defects</td>
<td>3 (1.2%)</td>
<td>169 (37%)</td>
<td>189 (30%)</td>
<td>240 (34%)</td>
<td>117 (27%)</td>
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<tr>
<td>Normal</td>
<td>237 (91.5%)</td>
<td>182 (40%)</td>
<td>199 (32%)</td>
<td>160 (23%)</td>
<td>155 (36%)</td>
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<td>Totalc</td>
<td>259</td>
<td>452</td>
<td>621</td>
<td>703</td>
<td>431</td>
</tr>
</tbody>
</table>

* Injection dose: Needle concentration of either antisense mir morpholino (M-1 or M-2) diluted in Danieau solution. M1 and M-2 represent morpholinos specific to coding and/or 5’ untranslated sequence outlined in Fig. 1. * Synergism of injecting both morpholinos at a needle concentration of 0.5 µg/µl each (total concentration of 1.0 µg/µl). * Embryos were considered as failing to complete gastrulation if they had an open yolk plug at 12 hpf. The majority of these embryos died, contributing to the increased mortality. b Pooled data from several clutches of embryos; does not include unfertilized embryos. (n), the number in brackets represent the percent relative to the total number of embryos.

Table 2

Effects of the control morpholino targeting human beta-globin on zebrafish embryogenesis

<table>
<thead>
<tr>
<th></th>
<th>Danieau 0.5 µg/µl*</th>
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<th>10 µg/µl</th>
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<tr>
<td></td>
<td>M-1</td>
<td>M-2</td>
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</tr>
<tr>
<td>Dead</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>19 (7.3%)</td>
<td>61 (16%)</td>
<td>41 (12%)</td>
</tr>
<tr>
<td>Gas. failurea</td>
<td>1</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Trunk and Tail defects</td>
<td>3 (1.3%)</td>
<td>12 (3%)</td>
<td>6 (2%)</td>
</tr>
<tr>
<td>Normal</td>
<td>237 (91.5%)</td>
<td>320 (81%)</td>
<td>308 (87%)</td>
</tr>
<tr>
<td>Totala</td>
<td>259</td>
<td>393</td>
<td>356</td>
</tr>
</tbody>
</table>

* Injection dose: Needle concentration of the control morpholino targeting human beta-globin. a Embryos were considered as failing to complete gastrulation if they had an open yolk plug at 12 hpf. The majority of these embryos died, contributing to the increased mortality. a Pooled data from several clutches of embryos; does not include unfertilized embryos. (n), the number in brackets represent the percent relative to the total number of embryos.

Table 3

Rescue of Mir morphants by coinjection with full-length Mir

<table>
<thead>
<tr>
<th></th>
<th>5.0 µg/µl</th>
<th>5.0 µg/µl M-2</th>
<th>5.0 µg/µl M-2</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>vector</td>
<td>+ M-2</td>
<td>+ M-2</td>
</tr>
<tr>
<td>Dead</td>
<td>256 (62.4%)</td>
<td>221 (57%)</td>
<td>136 (31%)</td>
</tr>
<tr>
<td>Gas. failurea</td>
<td>63</td>
<td>85</td>
<td>48</td>
</tr>
<tr>
<td>Trunk and Tail defects</td>
<td>128 (31.2%)</td>
<td>120 (31%)</td>
<td>58 (13%)</td>
</tr>
<tr>
<td>Normal</td>
<td>26 (6.4%)</td>
<td>45 (12%)</td>
<td>242 (56%)</td>
</tr>
<tr>
<td>Totala</td>
<td>410</td>
<td>386</td>
<td>436</td>
</tr>
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* Injection dose: Needle concentration of the control morpholino targeting human beta-globin. a Embryos were considered as failing to complete gastrulation if they had an open yolk plug at 12 hpf. The majority of these embryos died, contributing to the increased mortality. a Pooled data from several clutches of embryos; does not include unfertilized embryos. (n), the number in brackets represent the percent relative to the total number of embryos.
Even its name, Mir, is confusing given that it also binds Annexin V and possibly other proteins (M.N.K., unpublished observations). To date there have been only three Band 4.1 members reported in zebrafish, Nbl4 (Kelly and Reversade, 1997), Myosin VIIa (Ernest et al., 2000), and the founder 4.1R (Shafizadeh et al., 2002). Like mir, nbl4 is also expressed maternally and ubiquitously throughout development. This similarity in expression profile and the presence of the hallmark FERM domain may be the only features shared by zebrafish Mir and Nbl4. Results from our morpholino knock-down experiments show that other members of the Band 4.1 family do not compensate the loss of Mir. Thus, the loss of Mir activity leads to death and indicates that the expression of this protein is essential for development. The fact that the gene is expressed in embryos prior to the appearance of distinct germ layers would also indicate that Mir functions in undetermined cells, and its role may depend on the formation of multimeric complexes involving myosin regulatory light chain, Annexin V, and possibly other yet to be identified proteins.

Annexin V is often used as a marker to detect cells undergoing apoptosis. It is a ubiquitously expressed protein that can form voltage-gated calcium channels, act as an anticoagulant and as an inhibitor of protein kinase C-mediated phosphorylation of myosin light chain kinase substrates (Funakoshi et al., 1987; Schlaepfer et al., 1992; Demange et al., 1994; Dubois et al., 1998). In vitro assays show that Annexin V binds to the phospholipid bilayer in the plasma membrane (Isa et al., 2000), where it acts as a voltage-gated calcium channel (Berendes et al., 1993). This intracellular calcium regulating activity accounts for its ability to promote survival of T-lymphocytes (Gidon-Jeangirard et al., 1999). Annexin V also binds synaptic vesicles, early and late endosomes, and cytoskeletal structures, and it has been implicated in exocytosis (Koster et al., 1993; Gotow et al., 1996; Diakonova et al., 1997). Others have reported that it localizes to the nucleus where it interacts with helicase and demethylase (Ohsawa et al., 1996), or to the actin-based cytoskeleton near the plasma membrane (Tzima et al., 1999). Recently, a detailed analysis of annexin V expression during mouse embryogenesis showed that the protein is a marker of specific cell lineages associated with the developing vasculature and skeletal system (Brachvogel et al., 2001). To date, however, the exact role(s) for Annexin V in embryogenesis has not been reported.

The plethora of functions and their diverse nature indicate that Annexin V has multiple roles depending on where it is localized in the cell. Therefore, disrupting its localization is likely to have profound effects on its ability to perform one, if not all, of its normal roles. The question remains as to whether or not Mir, through one or more of its protein–protein interacting motifs, facilitates the translocation and subsequent function of Annexin V in the cell. Given the evidence that Annexin V associates with early and late endosomes (Diakonova et al., 1997) and the fact that it, together with Mir, colocalizes with the early endosomal marker Rab5 (unpublished observations), it is tempting to hypothesize that Mir has a role in vesicle trafficking. The mechanisms of endosome fusion and motility and their roles in influencing tyrosine kinase receptor trafficking are well documented (Clague and Urbe, 2001). In relation to zebrafish development, growth factors that bind these receptors, including EGF and EGF-related molecules, are essential for correct signaling during gastrulation, leading to the formation of mesoderm and endoderm (Saloman et al., 2000; Shen and Schier, 2000). Similarly, the endocytosis of C-cadherin was reported as necessary for gastrulation in Xenopus (Jarrett et al., 2002). If our hypothesis is correct and the retardation of cell movements during gastrulation in mir-morpholino-injected embryos is due to the disruption of vesicle trafficking and tyrosine kinase signaling or cadherin-mediated adhesion, then blocking Annexin V translation should phenocopy the Mir morphants. Work is currently in progress to test whether or not Mir, together with Annexin V, is involved in receptor trafficking and endosome motility.

To address the Mir-Annexin V relationship, attempts were made to identify Mir dominant negatives or positives that would scavenge endogenous Annexin V in embryos. Defects seen due to overexpressing Mir constructs, together with the yeast two-hybrid results, indicate that, while the full-length polypeptide is required to bind Annexin V, it is the deletion of the RING finger that perturbs development. This would indicate that the function of Mir, and more specifically the RING finger, depends on the interaction(s) with protein(s) other than Annexin V. Interestingly and unexpectedly, was the fact that overexpressing full-length Mir in intact embryos had no effect, despite the previous report showing that the overexpression of human MIR in PC12 cells blocks neurite outgrowth (Olsson et al., 1999). A number of factors, including the notion that steady-state protein levels were not altered in the embryo or there is rapid turnover of exogenous Mir, could explain this apparent contradiction. Likewise, previous studies have noted that overexpression of a protein in tissue culture cells may not always complement results seen using intact, rapidly developing embryos (Mangos et al., 2001). Thus, to circumvent the problems associated with overexpressing Mir in embryos, the logical approach was to deplete Mir and show that its loss perturbs embryogenesis.

In developing vertebrate oocytes and embryos, morpholino antisense oligos are effective in blocking translation of a specific protein (Summerton, 1999; Ekker and Larson, 2001; Vanderbeld and G.M.K., unpublished observations). Using this approach, we demonstrated that 1–4 cell-stage embryos injected with mir morpholinos cleave normally and develop, but only later do we see morphant phenotypes. These defects in cells derived from multiple germ layers were expected given the ubiquitous expression of mir mRNA. Disrupting cell motility associated with epiboly, convergence, and extension movements of gastrulation has severe consequences on subsequent cell differentiation and
As the enveloping layer spreads over the embryo, the deep cells undergo involution followed by convergence and extension to form the embryo proper. The mechanism(s) underlying these movements is not fully understood, but is driven by noncanonical Wnt signaling. These signals direct not only the direction but speed of cell motility and are interconnected with specification. In this way, cell migration and morphogenesis are linked. Disrupting cell migration has severe consequences on subsequent cell differentiation and development since misplaced cells required to complete normal embryogenesis are inappropriately positioned to signal. For instance, the overexpression of slit2 in zebrafish embryos results in head defects such as cyclopia as prechordal mesoderm is shifted away from the eye field (Piper and Little, 2003). Similarly, the failure of convergence and extension due to mutations in components of the noncanonical Wnt signaling pathways shortens the antero-posterior axis while broadening the embryos mesolaterally (reviewed in Myers et al., 2002). Mesodermal structures that depend on extension, such as the notochord, are often shortened, while somites and axial tissues that exhibit a large degree of convergence are broadened. For example, morpholino knock-down of Frizzled 2 in zebrafish embryos delays convergence and extension movements resulting in severe dorsal defects, such as oblong shaped embryos with undulating notochords (Sumanas et al., 2001). These gastrulation defects translate into a shortened body axis and tail defects. Similar defects are observed when embryos are depleted of Mir.

Embryos depleted of Mir exhibit delays in gastrulation, most noticeable in the retardation and sometimes failure of the enveloping layer to cover the yolk mass. This is evident in the extent of krox20 staining in morphants relative to control embryos. In addition to epiboly defects, morphants characteristically show delays in convergence and extension. In our study, while cell and tissue positions are altered germ layer differentiation is not. Components of neural ectoderm are normally positioned along the midline through appropriate convergence and differentiation (Steit, 2002). Mir knock-down leads to delays not only in the convergence of the neural ectoderm that contributes to rhombomere 3 and 5 formation, but also in delays in extension that serve to separate the rhombomeres. Similarly, mesoderm forming the notochord, paraxial tissue, and somites is compressed along the anteroposterior axis while showing mesolateral expansion. These defects are similar to pipetail/wnt5 mutants in zebrafish (Kilian et al., 2003). Pipetail mutants...
Fig. 11. *In situ* hybridization analysis shows that the delay in epiboly caused by the mir morpholino results in patterning defects in neural- and mesodermal-derived tissues at 14 hpf. (A) In a control embryo, *krox20* appears as two distinct doublets corresponding to rhombomeres 3 and 5 of the hindbrain. (B) In a morpholino-injected embryo, there are two regions of *krox20* expression, but instead of being localized distinctly in the hindbrain as in the embryo in (A), the signals are neither separated in the anteroposterior direction nor have they converged over the embryonic midline. The positioning of the *krox20*-positive bands in the morphant reflects a delay in epiboly. (C) The expression of the mesodermal marker *ntl* clearly denotes the gradual curvature of the notochord in a control embryo. (D) Although the expression of *ntl* in a mir-morpholino-injected embryo remains confined to the notochord, the obvious shape of the notochord indicates that patterning defects had occurred. (Inset) In contrast, severe morphants display an open yolk plug (asterisk) and have compressed *ntl* staining in the posterior region. Note that in controls the notochord continues around the curvature of the yolk and beyond the plane of view. In morphants, however, the notochord does not curve over the yolk mass, but instead ends abruptly within the plane of focus. (E) The expression of *myoD* is localized to developing somites in this control embryo. (F) *MyoD* staining in the Mir morphant is irregular and the expression in the somites obviously different form that in the control embryo seen in (E). The inset shows a severe morphant with somites compressed in the posterior orientation, but expanded mesolaterally. (G) Control embryo double stained for *hgg-1* and *dlx-3* shows the *hgg-1* signal in an arrowhead shape denoting the prechordal plate mesoderm. The overlapping expression pattern of *dlx-3* in the neurectoderm extends the entire length of the embryo. (H) In morpholino-injected embryo expression of *hgg-1* and staining of *dlx-3* in the anterior region appears normal. *dlx-3* expression in more posterior regions has expanded relative to that seen in the control (G) and thus indicates a delay in convergence.
able to perform its normal role(s) during early embryogenesis. Subsequent replacement of new Mir, either from maternal stores of mRNA or from zygotically transcribed mRNA after the midblastula transition would be blocked by the morpholino. We propose that this lag between protein availability and replacement accounts for the cell migration defects that appear following a turnover of maternal Mir. Antibodies directed against zebrafish Mir are being generated in order to test this hypothesis.

In conclusion, we have identified the zebrafish homolog of the human MIR protein, have characterized its expression during embryogenesis, and have shown that its expression is absolutely required for normal development. Little is known about the function of MIR in humans, although it was reported to play a role in neural cell motility and neurite outgrowth (Olsson et al., 1999 and 2000). Whether the depletion of zebrafish Mir perturbs vesicle trafficking required for correct growth factor signaling, adhesion, or the calcium-dependent phosphorylation and subsequent activation of myosin-dependent processes necessary during early embryogenesis, or all three, are avenues that are under investigation. These and other studies are ongoing to determine whether Mir functions in Wnt-mediated gastrulation movements in vertebrate embryos.

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Note added in proof. Sequence and expression data for Annexin V was recently published by Faber et al., 2003.

References


