1 **Characterization of the three zebrafish orthologs of the mitochondrial GTPase Miro/Rhot**

3 **Brittany M. Hollister^a, Kelsey A. Oonk^b, Douglas C. Weiser^c, and Susan Walsh^{d*}**

5 *a. Vanderbilt Genetics Institute, Vanderbilt University Medical Center, Nashville, TN 37232-*
6 *2358, USA*

7 *b. Department of Cell Biology, Duke University, Durham, NC 27710, USA*

8 *c. Department of Biological Sciences, University of the Pacific, Stockton, CA, 95211, USA*

9 *d. Department of Biology, Rollins College, Winter Park, FL 32789, USA*

12 **Running title:** Mitochondrial Rhot in zebrafish

14 **ms. has 37 pages, 10 figures, and 4 tables**

16 * Corresponding author:

17 Dr. Susan Walsh

18 Department of Biology

19 Rollins College

20 1000 Holt Ave – 2743

21 Winter Park, FL 32789, USA

22 407-646-2534

23 sjwalsh@rollins.edu

Abstract

Mitochondria exhibit dynamic locomotion and spatial rearrangement. This movement is necessary for a cell to maintain basic metabolic functions, and disruption of motility often results in cell death. Miro is a mitochondrial outer membrane Rho GTPase essential for mitochondrial movement and distribution in diverse systems, including yeast, animals, and plants. We sought to study the previously uncharacterized Miro protein family in zebrafish. We confirmed that, like human Miro, the zebrafish Miro proteins (Rhot1a, Rhot1b, and Rhot2) localize to mitochondria in mammalian tissue culture cells by both biochemical fractionation and immunofluorescent colocalization. In addition, using whole mount in situ hybridization, we observed ubiquitous expression of all three mRNAs throughout development. By microinjecting three antisense morpholino oligonucleotides targeted to each of the *rhot* genes, we knocked down all three proteins simultaneously in developing zebrafish embryos. The triple morphants demonstrated a dose-dependent defect in posterior body-axis elongation, while a single knockdown of each protein at the same dose produced no effect. This phenotype could be rescued with human *Miro1* mRNA and is most likely due to increased cell death. Taken altogether, this research demonstrates the importance of the Rhot proteins during vertebrate development.

Key Words: Rhot, Miro, mitochondria, zebrafish, Trak, *Danio rerio*

Abbreviations: KIF5 (kinesin superfamily protein 5), KHC (kinesin heavy chain), Miro (mitochondrial Rho), Trak (trafficking protein, kinesin binding), PINK1 (PTEN-induced kinase 1), Tom40 (translocase of the outer membrane 40), UTR (untranslated region), dpf (days post fertilization), MO (morpholino oligonucleotide), hpf (hours post fertilization)

1. Introduction

Mitochondria are dynamic organelles responsible for a variety of functions, including producing ATP, buffering calcium, and signaling apoptosis. In order to meet the needs of a cell, mitochondria must be mobile to perform these tasks within localized regions of the cell. This motility is mediated by complexes of motor proteins, adaptor proteins, and cytoskeletal elements (reviewed in Cai et al., 2011; Saxton and Hollenbeck, 2012). Miro (mitochondrial Rho) is a highly conserved mitochondrial outer membrane protein found on both stationary and motile mitochondria (Macaskill et al., 2009; Salas-Vidal et al., 2005). In flies and mammals, a protein complex composed of Miro, Milton/Trak, and kinesin regulates microtubule-based transport (reviewed in Sheng, 2014). Specifically, Miro and Milton/Trak are adaptors for the KIF5 kinesin heavy chain (KHC) to facilitate mitochondrial movement along microtubules (Wang and Schwarz, 2009). While Milton is the adaptor protein for Miro in *Drosophila*, the orthologs Trak1 and Trak2 link kinesin to Miro1 and Miro2 in mammals and are necessary for proper mitochondrial motility (Koutsopoulos et al., 2010).

The importance of Miro has been demonstrated in several model systems. For example, in *Drosophila*, the loss of Miro-dependent mitochondrial movement causes a depletion of mitochondria from the dendrites and axons of neurons, hindering the synaptic transmission across the neuromuscular junction (Guo et al., 2005). Furthermore, mutation of Miro causes premature lethality and arrested development at the pupal stage, including preventing mitochondrial transport into axons and dendrites and increasing fragmentation of mitochondria within the cells (Babic et al., 2015). In *Arabidopsis*, Miro1 is required for embryogenesis, and mutants in Miro1 demonstrate abnormal mitochondrial distribution and impaired pollen germination (Yamaoka et al., 2011). In *Saccharomyces*, the loss of Miro causes abnormal

mitochondrial morphology and slow growth on glycerol media, indicative of reduced respiratory function (Frederick et al., 2004). Similar phenotypes have been seen in mammalian cells that have lost or mutated Miro (Fransson et al., 2003, 2006; Macaskill et al., 2009; Saotome et al., 2008). In mice, loss of Miro1 resulted in animals that died shortly after birth because they were unable to expand their lungs due to defects in brainstem cranial neurons (Nguyen et al., 2014). Therefore, a neuron-specific conditional Miro1 knockout mouse was made. These mice present a phenotype similar to human motor neuron diseases, suggesting that Miro1 is essential for the development of motor neurons required for movement (Nguyen et al., 2014). In addition, Miro is a substrate for the Parkin-dependent mitophagy process present in healthy neurons (Liu et al., 2012; Sarraf et al., 2013; Weihofen et al., 2009).

Zebrafish (*Danio rerio*) are frequently employed as models for human mitochondrial dysfunction (reviewed in Steele et al., 2014), and zebrafish disease models have been generated using antisense morpholino oligonucleotides (MOs) targeted to mitochondrial proteins: for example, morphants in *tomm22*, *coa6*, and *opal* (Curado et al., 2010; Ghosh et al., 2014; Rahn et al., 2013). External fertilization and development also permit relatively easy drug testing for pharmaceuticals which affect mitochondrial proteins (Bestman et al., 2015; Dabir et al., 2013; Pinho et al., 2013). Although the Miro proteins play a critical role in a number of model systems, the zebrafish orthologs of *miro* (*rhot1a*, *rhot1b*, and *rhot2*) are largely unexplored. Zebrafish are an ideal model as they have a short generation time and produce a large number of transparent embryos. In contrast to mice, Miro protein can be knocked down in a whole embryo to directly assess phenotypes. The accessibility of the embryos enabled us to study how Miro affects the development of a zebrafish embryo. Although we initially expected a difference among the three *miro* orthologs, suggesting a divergence of function or expression, we found

instead that the *rhot* orthologs are expressed similarly throughout development and only depletion of all three proteins results in significant defects in development. In addition, biochemically, the Rhot proteins behaved in a similar fashion as the human protein equivalents by localizing to the mitochondria, although there are some differences in the paralogs ability to interact with human Trak. Taken altogether, we propose that zebrafish may be a good noninvasive developmental model for future mitochondrial trafficking studies.

2. Experimental Procedures

2.1. Zebrafish (*Danio rerio*) Manipulations

All animals were raised in an Institutional Animal Care and Use Committee-approved facility at Rollins College. Adult zebrafish (TL from ZIRC or WT from Aquatic Research Organisms) were fed brine shrimp daily. In order to prepare embryos for microinjection, males and females were placed on opposite sides of a divider in a breeding tank overnight. After removing the divider, embryos were collected 20 minutes after breeding. Healthy embryos were dechorionated using pronase (20 mg/mL; Sigma Aldrich) and washed with approximately 500 mL H buffer (0.12 M NaCl, 4.8 mM NaHCO₃, 2.2 mM CaCl₂, and 1.3 mM KCl, pH 6.6-7.0) to remove the pronase. Approximately half of the healthy embryos were saved as uninjected controls. Embryos were injected at the single-cell stage with a single antisense morpholino oligonucleotide (MO), co-injected with all three AMOs, or co-injected with all three AMOs and myc-hMiro1 mRNA (225 pg) in a total volume of 1 nL (Table 1). Embryos were grown in a glass petri dish filled with H buffer at 28.5°C. After shield stage, all embryos were transferred into 1X E3 buffer (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄). Viable phenotypes were determined at 1 day post fertilization (dpf). Data significance was determined

using one-way ANOVA followed by a Tukey test. Acridine orange staining was performed on 1 dpf embryos (Tucker and Lardelli, 2007).

For western blots, approximately 20 embryos were collected at 1 dpf. Yolk lipids were removed by pipetting up and down in 200 μ L deysolking buffer (55 mM NaCl, 1.8 mM KCl, 1.25 mM NaHCO₃) and vortexing on low for 5 minutes. Embryos were spun at 300xg for 5 min at 4°C. The cell pellet was then washed twice in 500 μ L wash buffer (110 mM NaCl, 3.5 mM KCl, 2.7 mM CaCl₂, 10 mM Tris-HCl pH 8.5). Cells were resuspended in 2 μ L SDS-PAGE sample buffer per embryo. c-myc antibody (1:2000; sc-40; Santa Cruz Biotechnology) or Rho T2 antibody (1:100; H-45; Santa Cruz Biotechnology) were used to detect protein expression after microinjection of myc-hMiro1 mRNA or the MOs.

Table 1. Antisense Morpholino Oligonucleotides

Target	Sequence	Concentration
Rhot1a	ATAAAAAACGGACGAGTCCTTTGTC	0.75 ng
Rhot1b	TTTTCCTACTTCTGAAGACAGATCAC	4 ng
Rhot2	CCGGACATCGCGTTTCATTTCAGTC	4 ng

2.2. DNA Constructs and PCR

Zebrafish mRNA at various stages was extracted using TRIzol (Invitrogen). Zebrafish cDNA (3 dpf) was then synthesized using random hexamers and the SuperScriptIII kit (Invitrogen). DNA sequences were amplified using OneTaq (NEB) and cloned into the appropriate vectors using the indicated restriction enzymes (Table 2). For myc-tagged expression, coding regions were cloned into pcGlobin2 (Ro et al., 2004) with a N-terminal myc tag in-frame; for in situ probes, 3'UTRs were cloned into pCR4 TOPO (Invitrogen) or pCS2+; for yeast two-hybrid, hMiro1 and Rhos without the transmembrane domain were cloned into pGBKT7 (Clontech) and hTraks were cloned into pGADT7 (Clontech). For all but Rhot1b and Rhot2, these constructs were made using primers that had a stop codon before the

transmembrane domain and cloning a PCR product. For Rhot1b and Rhot2, the premature stop codons were generated using Quikchange site-directed mutagenesis (Agilent) and then cloned into pGBKT7 using EcoRI and XhoI. The 3' UTR for Rhot1a was 325bp, for Rhot1b 303bp, and for Rhot2 305bp. For RT-PCR experiments, similar amounts of starting total RNA were used to generate cDNA, and an equal volume of cDNA was used in a PCR reaction for 30 cycles. Myc-tagged hMiro1 and hMiro2 were in pRK5-myc vectors (gift from G. Hajnóczky, Thomas Jefferson University). Templates for amplifying hTrak1 and hTrak2 were provided by M.T. Ryan (Monash University). Primers for β -actin were 5'-ATCAGGGTGTCATGGTTGGT and 5'-CACGCAGCTCGTTGTAGAAG.

Table 2. Primers.

3' UTRs for in situ	
Rhot1a Forward.BamHI	5'- GGATCC GGTGATCAGTGCAAATAC
Rhot1a Reverse.XhoI	5'- CTCGAG CCTATCAGGCTTCTCTGC
Rhot1b Forward.BamHI	5'- GGATCC ACGGTTCGGACCAAATC
Rhot1b Reverse.XhoI	5'- CTCGAG TCTCAAGACTTGGGAATG
Rhot2 Forward.BamHI	5'- GGATC CTCATGGCATGAGCCCCG
Rhot2 Reverse.XhoI	5'- CTCGAG CAAGACAGCGCAGAAAATG
Full length coding region	
Rhot1a Forward.EcoRI	5'- GGAATTC ATGAGAAAGGACGTGAGG
Rhot1a Reverse.NotI	5'- TGCGGCCG CATCACCGCTGCTTGAGG
Rhot1b Forward.EcoRI	5'- GGAATTC ATGCGAAAGGACGTGAG
Rhot1b Reverse.XhoI	5'- TCTCGAG TCATCGGTTTCTGAGGAG
Rhot2 Forward.EcoRI	5'- GGAATTC ATGAAACGCGATGTCCG
Rhot2 Reverse.XhoI	5'- ACTCGAG TCATTTGTGTCGGCTAAAG
Yeast Two Hybrid	
Rhot1a Forward.EcoRI	5'- GGAATTC ATGAGAAAGGACGTGAGG
Rhot1a Reverse.SalI	5'- CAGGTCG ACTCAAAATGTAGAGTTTTTCAGGT
Rhot1b Forward.stop	5'-GAAAAACTCTTCACTTTGACTCAAAGCGAGCGTTGG
Rhot1b Reverse.stop	5'-TCCAACGCTCGCTTTGAGTCAAAGTGAAGAGTTTTTC
Rhot2 Forward.stop	5'-CGTCTTTCTGGCTTCGTTGACTCTTGGAGCCA
Rhot2 Reverse.stop	5'-TGGCTCCAAGAGTCAACGAAGCCAGAAAGA
hMiro1.Forward.EcoRI	5'- GATGAATTC ATGAAGAAAGACGTGCG
hMiro1.Reverse.SalI	5'- CATGTCG ACAAAACGTGGAGCTCTTG
hMiro2.Forward.EcoRI	5'- GCCGAATTC ATGAGGCGGGACGTGC
hMiro2.Reverse.XhoI	5'- CAGCTCG AGTACCGGAGCCAGAAGGAAGA

hTrak1.Forward.BamHI	5'-CAAGGATCCTCATGGCATTGGTTTTTCAATTCGG
hTrak1.Reverse.XhoI	5'-CAACTCGAGTCACCGTAAGCTAGTTTG
hTrak2.Forward.ClaI	5'-GCAATCGATACATGAGTCAATCCCAGAATGC
hTrak2.Reverse.BamHI	5'-CAAGGATCCTCAGTCCTCCTTCAGGACA

Restriction sites are bolded. For yeast-two-hybrid, Rhots and Miros are designed to stop at the transmembrane domain.

2.3. Whole Mount RNA In Situ Hybridization

In order to synthesize RNA probes for the Rhot 3'UTRs, the vector was linearized with either NcoI or PstI. The linearized DNA was extracted with phenol:chloroform and ethanol precipitated. The synthesis reactions included T3 (ThermoFischer) or T7 enzyme (NEB) and DIG-UTP labeling mix (Roche). Embryos were treated with a low dose of phenylthiourea (PTU) to inhibit pigmentation and processed according to standard whole mount in situ hybridization protocols (Thisse and Thisse, 2008). *Dlx3*, *shh*, and *pax2.1* probes were also used (Weiser and Kimelman, 2012).

2.4. Tissue Culture Manipulation

COS7 or HEK293T cells (ATCC) were cultured in DMEM with 10% FBS. COS7 cells were split onto glass coverslips into a 12 well plate prior to transfection and grown to 50%-80% confluence. Cells were transfected using Lipofectamine 2000 (Invitrogen). HEK293T cells were grown in 10cm dishes and transfected using Lipofectamine 2000. All cells were processed 20 to 24 hours post transfection.

For mitochondrial fractionation, approximately 8×10^6 cells were removed from the plate with PBS, pelleted, and incubated in 500 μ L 1X MB (0.5 M sucrose, 5 mM Tris pH 7.5, 80 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM EDTA, 1 mM DTT) with protease inhibitors on ice for 30 minutes. Cells were broken open gently using a tight-fitting B pestle 50 times. Unbroken material was pelleted out at 700xg for 10 minutes at 4°C. Mitochondria were pelleted from the

supernatant by spinning at 14000xg for 10 minutes at 4°C. Mitochondria were then washed in 750 µL of 0.5X MB by centrifugation at 11000xg for 10 minutes at 4°C. The mitochondria were resuspended in 0.5X MB, quantified by Bradford assay, and then equal amounts of protein were treated with 100 µg/mL Proteinase K, with 20 µg/mL Proteinase K and 0.1% Triton X, or untreated for 30 minutes on ice. Digestion was stopped with 5 mM PMSF, mitochondria were pelleted, and loaded on a 12% SDS-PAGE for western blotting for c-myc antibody (1:2000; sc-40; Santa Cruz Biotechnology) and Tom40 (ULAB4; 1:1000; gift of Carla M. Koehler, UCLA).

2.5. Immunofluorescence

Transfected COS7 cells were washed with 1X PBS and fixed with methanol. After washing with 1X PBS three times for five minutes per wash, cells were blocked in 0.3% Triton X, 5% goat serum, 1X PBS for one hour at room temperature. Primary antibodies myc (9E10; 1:500; Developmental Studies Hybridoma Bank) and Tom 40 (ULAB4; 1:500; gift of Carla M. Koehler, UCLA) were added, and the cells were incubated at 4°C overnight. After washing the cells three times with 1X PBS, fluorescent secondary antibodies were added and allowed to incubate for one hour at room temperature. Secondary antibody goat ant-rabbit Alexafluor 594 (Invitrogen) was used for Tom40, and goat anti-mouse Alexafluor 488 (Invitrogen) was used for 9E10 myc, both at 1:10000. The cells were DAPI stained for 5 minutes at room temperature. The cells were then washed twice with 1X PBS for 5 minutes each wash. Pictures were taken using a laser scanning confocal fluorescent microscope at a total magnification of 400x.

2.6. Yeast Two Hybrid

Yeast transformations employed the high-efficiency method (Gietz and Woods, 2002).

All pGADT7 constructs were transformed into Y187 (Clontech) and selected on SD-LEU while all pGBKT7 constructs were transformed into Y2HGold (Clontech) and selected on SD-TRP. Diploids were created by mating transformed haploids in 500 μ L of YPD at 30°C for approximately 24 hours and selecting for colonies that grew on SD-LEU-TRP plates. Diploids were then streaked onto both SD-LEU-TRP as a control for viability and SD-LEU-TRP-ADE-HIS with X- α -gal (40 μ g/mL) to test for a positive interaction.

3. Results and Discussion

3.1. The zebrafish Rhot genes are highly conserved

Zebrafish contain three Rhot genes: *rhot1a*, *rhot1b*, and *rhot2* (Figure 1A; GenBank accession numbers BC044431, XM_005166190, and NM_001037679.1, respectively). *Rhot1a* is located on chromosome 3, *rhot1b* on chromosome 6, and *rhot2* on chromosome 24 (www.zfin.org). It is not surprising that zebrafish have three genes as contrasted with only two in mammals, as this pattern is attributed to a genome duplication event (Meyer and Schartl, 1999; Taylor, 2003; Taylor et al., 2001; Woods et al., 2005). Although this model would predict four distinct genes, it is not uncommon to lose a gene through pseudogenization and less stringent selection on genes with redundant function (Meyer and Schartl, 1999). However, redundant paralogs may be maintained in a lineage for longer than expected, resulting in three instead of two genes (Meyer and Schartl, 1999; Taylor et al., 2001). Indeed, BLAST searches using the zebrafish genes against a number of sequenced fish species also identified three distinct genes in fifteen different species of fish (Table S1). Of the sequences compared, the Mexican tetra (*Astyanax mexicanus*), also of the Cypriniformes (Jeffery, 2001), shares a close common ancestor with zebrafish for each *rhot* (Figure S1).

Rhot1a and *rhot1b* cluster with the human and mouse *miro1* genes, while *rhot2* is more similar to *miro2* (Figure 1B). Each of the genes was identified based on their similarity to Rho small GTPases (Salas-Vidal et al., 2005) and contains two GTPase domains, two EF-hand domains, and a C-terminal transmembrane domain, like the human and *Drosophila* orthologs (Fransson et al., 2003, 2006; Guo et al., 2005; Klosowiak et al., 2013). Specifically, the P loop of the first GTPase domain is identical among all three Rhots. This demonstrates the importance of this domain to function, and indeed, in *Drosophila* Miro (dMiro), a point mutation in this region (T25N) causes lethality, mitochondrial abnormalities, and defects in mitochondrial locomotion, unlike mutation of the second GTPase domain (Babic et al., 2015). The fact that mutation of the second GTPase domain has less effect on viability and function provides a possible explanation why this region of the zebrafish Rhots is not strictly conserved. A salt bridge between R444 and D521 is important for contacts between the EF hand and C-terminal GTPase domain in dMiro (Klosowiak et al., 2013), and homologous residues, R415 and D491/490, also exist in the zebrafish Rhot proteins. The highly conserved C-terminal transmembrane domain is key for anchoring the protein in the mitochondrial outer membrane (Fransson et al., 2006) and is likely responsible for that property in zebrafish as well. In mammalian and *Drosophila* Miro, putative Parkin ubiquitylation sites have been identified, namely K194, R214, K411, and K572 (human numbering) (Klosowiak et al., 2013; Sarraf et al., 2013). The zebrafish genes also have these lysines and arginines in common, with the exception of the third site in Rhot2. Finally, at least one putative PINK1 phosphorylation site, S156, was positively identified in dMiro (Wang et al., 2011), and this site, S155, also is conserved in the zebrafish genes, suggesting that these genes may be susceptible to similar regulatory pathways important for mitophagy.

3.2. Zebrafish Rhot proteins associate with mitochondria

In order to test if the zebrafish Rhot proteins are capable of localizing to mitochondria, full-length myc-tagged genes were transfected into COS7 cells. Proteins were tagged at the N-terminus to not interfere with localization by blocking the putative C-terminal transmembrane domain. After immunofluorescence for both exogenously expressed proteins and the integral mitochondrial outer membrane protein Tom40, much like both hMiro1 and hMiro2, all three zebrafish Rhot proteins localized to the mitochondria (Figure 2A). In order to confirm the immunofluorescence data biochemically, the same constructs were transfected into HEK293T cells and mitochondria were isolated by fractionation. Western blotting for myc or Tom40 showed that the Rhots localize to mitochondria (Figure 2B). The decrease in signal after protease treatment suggests that the proteins are localized to the outer membrane rather than protected on the inside of mitochondria. Although Tom40 identifies the presence of mitochondria, it is not degraded until detergent is added because it is an integral membrane protein.

Given the difficulty of finding reliable antibodies for zebrafish proteins, we employed a heterologous approach using a yeast two-hybrid assay to determine if the zebrafish Rhot proteins can directly interact with human Trak (hTrak) proteins. *Rhot* or *hMiro* genes were cloned in-frame with the GAL4 binding domain, and *hTrak* genes were cloned in-frame with the GAL4 activation domain. In diploid yeast, positive interactions allow for growth on selective media and the production of the MEL1 enzyme which catalyzes the breakdown of X- α -gal, turning the yeast blue (Figure 3). None of the fusion proteins self-activated, as demonstrated by a lack of growth in diploids expressing only a single protein (Figure 3 and data not shown). Like hMiro1 and hMiro2, Rhot2 interacted strongly with both hTrak1 and hTrak2. Surprisingly, Rhot1b

interacted weakly with both hTrak1 and hTrak2, and Rhot1a did not interact at all with either hTrak. Rhot1a and Rhot1b are 77% identical, whereas Rhot2 is only 62% identical to Rhot1a and 57% identical to Rhot1b (Figure 1A). The disparity in the protein sequences may explain why Rhot2 interacts better in this assay. The most notable distinction between the amino acid sequences of the paralogs Rhot1a and Rhot1b is an additional forty-one amino acids just before the C-terminal transmembrane domain of Rhot1b (Figure 1A). This region may be helical or coiled (Drozdetskiy et al., 2015), allowing for greater protein interactions or increased protein stability as compared to Rhot1a, either of which would produce the stronger interaction results from the yeast-two hybrid. Furthermore, it may be that Rhot1a and Rhot1b do not interact as well with the human proteins, but may interact with zebrafish Trak proteins to modulate mitochondrial trafficking. Finally, we cannot rule out additional accessory proteins, perhaps including kinesin heavy chain, which may stabilize the Rhot-Trak interaction in vivo that are not represented in this in vitro assay which occurs in the nucleus.

3.3. Zebrafish Rhot proteins are necessary for early development

The preservation of three distinct genes with a conservation of function suggested that perhaps these proteins might have different expression patterns. Previous data generated in a large-scale screen demonstrated that *rhot1a* mRNA was not spatially restricted during early embryonic development (Thisse and Thisse, 2004). However, data examining the other forms of *rhot* were lacking, prompting us to examine these genes via RNA in situ hybridization (Figure 4B-D). The 3'UTR of each gene was isolated by PCR from whole embryo cDNA and used as a probe in the stages examined: shield, 1 dpf, and 2 dpf (Figure 4A). Although the data shown are not quantitative, they do demonstrate that we can expect each gene to be present at the stages

examined by in situ hybridization. Shield stage embryos have ubiquitous expression of each *rhot*. Although *rhot2* demonstrates the strongest signal, all three genes have expression throughout the body in embryos at 1 dpf. Though not spatially restricted, *rhot2* demonstrates strongest expression in the head, specifically in the mesencephalon, and in the fin bud at 2 dpf. *Rhot1a* expression also occurs throughout the body but is concentrated in the head and fin bud. *Rhot1b* expression is faint, but only occurs in the head. These data are consistent with transcriptome analysis where *rhot1b* has been identified in adult brain and *rhot1a* and *rhot2* have been expressed throughout the organism (Bastian et al., 2008).

Given the nearly ubiquitous expression of the *rhot* mRNAs, we sought to determine the effect of knocking down protein expression using ATG-targeted antisense morpholino oligonucleotides (MOs). Initially, we microinjected each embryo with a single MO. At doses of up to 8 ng of either *rhot1b* or *rhot2* or 2 ng of *rhot1a*, there was not a significant difference in the percentage of embryos that developed normally in comparison with sibling uninjected controls (data not shown). This is not surprising, since these proteins may be functionally redundant and they do not have distinct tissue-specific expression patterns. Importantly, when injected with a single MO, the embryos appeared relatively normal, suggesting that the MOs do not induce nonspecific, off-target effects that are sometimes associated with certain MOs. Injection with MOs targeted to *rhot1a* or *rhot1b* yielded embryos that were slightly smaller, but still maintained morphological characteristics equivalent to their uninjected siblings (Figure 5). However, when all three MOs were co-injected at lower individual concentrations (0.75 ng *rhot1a*, 4 ng *rhot1b*, and 4 ng *rhot2*), the triple morphants demonstrated a dose-dependent defect in posterior body-axis elongation and smaller heads, while a single MO at the same dose produced no effect (Figures 5 and S2, Table 3). At a higher dose of the triple MO (1.5 ng *rhot1a*, 8 ng *rhot1b*, and 8

ng *rhot2*), embryos failed to complete epiboly and died during gastrulation (data not shown). The defects in the triple morphants are not due to errors in convergent extension movements, as in situs for *shh* do not show significant changes in the width of the midline at bud stage (Figure S3; Weiser and Kimelman, 2012). Instead, we suggest that microinjection of all three morpholinos causes cell death, as evidenced by an increase in acridine orange staining at 24 hpf, particularly in the tail (Figure 6). Increased cell death as a result of abnormalities in mitochondrial trafficking and distribution, or more specifically changes in Miro proteins, are not unexpected and are consistent with data from other species (Babic et al., 2015; Fransson et al., 2003, 2006; Guo et al., 2005; Nguyen et al., 2014; Yamaoka et al., 2011). Importantly, the morphant phenotype was partially rescued by the co-microinjection of myc-tagged hMiro1 mRNA, which should not be a target of the MOs and does not produce the same phenotype when microinjected alone (Figure S4; Table 3). Rescue experiments with co-injection of mRNA are considered the standard control to demonstrate specificity of a MO phenotype (Bill et al., 2009).

Table 3. Triple morphants display abnormal phenotypes as compared to single morphants or rescued embryos.

Microinjection	Normal Phenotype	Total
Rhot1a MO	91 ± 7%	82
Rhot1b MO	86 ± 14%	261
Rhot2 MO	98 ± 3%	155
3 MO	21 ± 28% *	352
3 MO + hMiro1 mRNA	76 ± 13% **	101

The average percentage of embryos demonstrating a normal phenotype with the standard deviation among individual sample sets is in the middle column with the total number of embryos at right. Statistics were analyzed using one-way ANOVA followed by Tukey analysis. * indicates a significant difference as compared to uninjected controls. ** indicates a significant difference as compared to triple morphants.

4. Conclusions

The conservation of the Miro protein family in eukaryotes suggests that they appeared early during evolution and play a vital role in organismal development and cell survival

(Fransson et al., 2003; Reis et al., 2009; Salas-Vidal et al., 2005; Yamaoka and Hara-Nishimura, 2014). Indeed, mitochondrial distribution is essential to the health of the cell, and Miro/Rhot has been demonstrated to be at the core of this process in a number of model systems. Here, we present data from zebrafish to reinforce the importance of Miro. We investigated the three *rhot* genes with the hypothesis that three separate genes would permit a distinction in function or gene expression. However, this was not the case, and preservation of three genes with redundant function have been seen in other gene families in fish (Meyer and Scharl, 1999; Taylor et al., 2001). Interestingly, although the three zebrafish Rhot proteins are conserved, demonstrate similar expression patterns, and are most likely functionally redundant, they are not identical in their interactions with human Trak, the adaptor protein that connects kinesin to Miro. Whether the Rhot proteins will interact similarly with various zebrafish Trak proteins may provide insight into function of both the Rhot and Trak proteins in vivo, since the Trak proteins have been demonstrated to have nonredundant functions between paralogs in other species (Brickley and Stephenson, 2011) and thus may be more likely to display distinct functions or expression patterns among the zebrafish genes. Future studies could also compare morphant phenotypes to those generated by genome editing using CRISPR technology (Schulte-Merker and Stainier, 2014). Finally, it would ideal to determine if the Rhot proteins are subject to the same regulation as proposed for other systems, specifically within the realm of mitophagy (reviewed in Wager and Russell, 2013). In fact, zebrafish mutants in the Parkinson's related mitophagy pathway already exist (Anichtchik et al., 2008; Fett et al., 2010; Flinn et al., 2009, 2013; Xi et al., 2010) so generating *rhot* morphants in this background would be particularly intriguing. Further research on both mitochondrial trafficking and mitophagy in zebrafish would create additional

human disease models for spinal motor neuron disease, Parkinson's, and other neurodegenerative diseases.

Acknowledgements

This project was initially proposed by Erin Jimenez (Johns Hopkins University) in Carla M. Koehler's laboratory at UCLA. DCW was supported by NSF grant IOS-1121310. The myc-tagged human Miro1 and Miro 2 were gifts from G. Hajnóczky (Thomas Jefferson University) with permission from Pontus Aspenström (Karolinska Institutet). Templates for amplifying hTrak1 and hTrak2 were provided by M.T. Ryan (Monash University). Carla M. Koehler (UCLA) provided the Tom40 antibody. Courtney Reddig (University of Georgia) and Cecelia Harold (Albert Einstein College of Medicine) helped to generate some of the yeast two hybrid constructs. Sally Butterfield and Amanda Ferguson maintained the fish colony. Jay Pieczynski (Rollins College) helped with imaging.

References

- Anichtchik, O., Diekmann, H., Fleming, A., Roach, A., Goldsmith, P., Rubinsztein, D.C., 2008. Loss of PINK1 function affects development and results in neurodegeneration in zebrafish. *J. Neurosci.* 28, 8199–207. doi:10.1523/JNEUROSCI.0979-08.2008
- Babic, M., Russo, G.J., Wellington, a. J., Sangston, R.M., Gonzalez, M., Zinsmaier, K.E., 2015. Miro's N-Terminal GTPase Domain Is Required for Transport of Mitochondria into Axons and Dendrites. *J. Neurosci.* 35, 5754–5771. doi:10.1523/JNEUROSCI.1035-14.2015
- Bastian, F., Parmentier, G., Roux, J., Moretti, S., Laudet, V., Robinson-Rechavi, M., 2008. Data Integration in the Life Sciences, Lecture Notes in Computer Science. Springer Berlin Heidelberg, Berlin, Heidelberg. doi:10.1007/978-3-540-69828-9
- Bestman, J.E., Stackley, K.D., Rahn, J.J., Williamson, T.J., Chan, S.S.L., 2015. The cellular and molecular progression of mitochondrial dysfunction induced by 2,4-dinitrophenol in developing zebrafish embryos. *Differentiation*. 89, 51–69. doi:10.1016/j.diff.2015.01.001
- Bill, B.R., Petzold, A.M., Clark, K.J., Schimmenti, L.A., Ekker, S.C., 2009. A primer for morpholino use in zebrafish. *Zebrafish* 6, 69–77. doi:10.1089/zeb.2008.0555
- Brickley, K., Stephenson, F.A., 2011. Trafficking kinesin protein (TRAK)-mediated transport of mitochondria in axons of hippocampal neurons. *J. Biol. Chem.* 286, 18079–92. doi:10.1074/jbc.M111.236018
- Cai, Q., Davis, M.L., Sheng, Z.-H., 2011. Regulation of axonal mitochondrial transport and its impact on synaptic transmission. *Neurosci. Res.* 70, 9–15. doi:10.1016/j.neures.2011.02.005
- Curado, S., Ober, E. a, Walsh, S., Cortes-Hernandez, P., Verkade, H., Koehler, C.M., Stainier, D.Y.R., 2010. The mitochondrial import gene tomm22 is specifically required for hepatocyte survival and provides a liver regeneration model. *Dis. Model. Mech.* 3, 486–95. doi:10.1242/dmm.004390
- Dabir, D. V, Hasson, S.A., Setoguchi, K., Johnson, M.E., Wongkongkathep, P., Douglas, C.J., Zimmerman, J., Damoiseaux, R., Teitell, M.A., Koehler, C.M., 2013. A small molecule inhibitor of redox-regulated protein translocation into mitochondria. *Dev. Cell* 25, 81–92. doi:10.1016/j.devcel.2013.03.006
- Drozdetskiy, A., Cole, C., Procter, J., Barton, G.J., 2015. JPred4: a protein secondary structure prediction server. *Nucleic Acids Res.* 43, W389–94. doi:10.1093/nar/gkv332
- Fett, M.E., Pilsl, A., Paquet, D., van Bebber, F., Haass, C., Tatzelt, J., Schmid, B., Winklhofer, K.F., 2010. Parkin is protective against proteotoxic stress in a transgenic zebrafish model. *PLoS One* 5, e11783. doi:10.1371/journal.pone.0011783

405 Flinn, L., Mortiboys, H., Volkmann, K., Köster, R.W., Ingham, P.W., Bandmann, O., 2009.
 406 Complex I deficiency and dopaminergic neuronal cell loss in parkin-deficient zebrafish
 407 (Danio rerio). *Brain* 132, 1613–23. doi:10.1093/brain/awp108

408 Flinn, L.J., Keatinge, M., Breaud, S., Mortiboys, H., Matsui, H., De Felice, E., Woodroof, H.I.,
 409 Brown, L., McTighe, A., Soellner, R., Allen, C.E., Heath, P.R., Milo, M., Muqit, M.M.K.,
 410 Reichert, A.S., Köster, R.W., Ingham, P.W., Bandmann, O., 2013. TigarB causes
 411 mitochondrial dysfunction and neuronal loss in PINK1 deficiency. *Ann. Neurol.* 74, 837–
 412 47. doi:10.1002/ana.23999

413 Fransson, A., Ruusala, A., Aspenström, P., 2003. Atypical Rho GTPases Have Roles in
 414 Mitochondrial Homeostasis and Apoptosis. *J. Biol. Chem.* 278, 6495–6502.
 415 doi:10.1074/jbc.M208609200

416 Fransson, S., Ruusala, A., Aspenström, P., 2006. The atypical Rho GTPases Miro-1 and Miro-2
 417 have essential roles in mitochondrial trafficking. *Biochem. Biophys. Res. Commun.* 344,
 418 500–10. doi:10.1016/j.bbrc.2006.03.163

419 Frederick, R.L., McCaffery, J.M., Cunningham, K.W., Okamoto, K., Shaw, J.M., 2004. Yeast
 420 Miro GTPase, Gem1p, regulates mitochondrial morphology via a novel pathway. *J. Cell*
 421 *Biol.* 167, 87–98. doi:10.1083/jcb.200405100

422 Ghosh, A., Trivedi, P.P., Timbalia, S.A., Griffin, A.T., Rahn, J.J., Chan, S.S.L., Gohil, V.M.,
 423 2014. Copper supplementation restores cytochrome c oxidase assembly defect in a
 424 mitochondrial disease model of COA6 deficiency. *Hum. Mol. Genet.* 23, 3596–606.
 425 doi:10.1093/hmg/ddu069

426 Gietz, R.D., Woods, R.A., 2002. Transformation of yeast by lithium acetate/single-stranded
 427 carrier DNA/polyethylene glycol method. *Methods Enzymol.* 350, 87–96.

428 Guo, X., Macleod, G.T., Wellington, A., Hu, F., Panchumarthi, S., Schoenfield, M., Marin, L.,
 429 Charlton, M.P., Atwood, H.L., Zinsmaier, K.E., 2005. The GTPase dMiro is required for
 430 axonal transport of mitochondria to Drosophila synapses. *Neuron* 47, 379–93.
 431 doi:10.1016/j.neuron.2005.06.027

432 Jeffery, W.R., 2001. Cavefish as a model system in evolutionary developmental biology. *Dev.*
 433 *Biol.* 231, 1–12. doi:10.1006/dbio.2000.0121

434 Klosowiak, J.L., Focia, P.J., Chakravarthy, S., Landahl, E.C., Freymann, D.M., Rice, S.E., 2013.
 435 Structural coupling of the EF hand and C-terminal GTPase domains in the mitochondrial
 436 protein Miro. *EMBO Rep.* 14, 968–74. doi:10.1038/embor.2013.151

437 Koutsopoulos, O.S., Laine, D., Osellame, L., Chudakov, D.M., Parton, R.G., Frazier, A.E., Ryan,
 438 M.T., 2010. Human Mitons associate with mitochondria and induce microtubule-dependent
 439 remodeling of mitochondrial networks. *Biochim. Biophys. Acta* 1803, 564–74.
 440 doi:10.1016/j.bbamcr.2010.03.006

441 Liu, S., Sawada, T., Lee, S., Yu, W., Silverio, G., Alapatt, P., Millan, I., Shen, A., Saxton, W.,
 442 Kanao, T., Takahashi, R., Hattori, N., Imai, Y., Lu, B., 2012. Parkinson's Disease-
 443 Associated Kinase PINK1 Regulates Miro Protein Level and Axonal Transport of
 444 Mitochondria. *PLoS Genet.* 8, e1002537. doi:10.1371/journal.pgen.1002537

445 Macaskill, A.F., Rinholm, J.E., Twelvetrees, A.E., Arancibia-Carcamo, I.L., Muir, J., Fransson,
 446 A., Aspenstrom, P., Attwell, D., Kittler, J.T., 2009. Miro1 is a calcium sensor for glutamate
 447 receptor-dependent localization of mitochondria at synapses. *Neuron* 61, 541–55.
 448 doi:10.1016/j.neuron.2009.01.030

449 McWilliam, H., Li, W., Uludag, M., Squizzato, S., Park, Y.M., Buso, N., Cowley, A.P., Lopez,
 450 R., 2013. Analysis Tool Web Services from the EMBL-EBI. *Nucleic Acids Res.* 41, W597–
 451 600. doi:10.1093/nar/gkt376

452 Meyer, A., Scharl, M., 1999. Gene and genome duplications in vertebrates: the one-to-four (-to-
 453 eight in fish) rule and the evolution of novel gene functions. *Curr. Opin. Cell Biol.* 11, 699–
 454 704. doi:10.1016/S0955-0674(99)00039-3

455 Nguyen, T.T., Oh, S.S., Weaver, D., Lewandowska, a., Maxfield, D., Schuler, M.-H., Smith,
 456 N.K., Macfarlane, J., Saunders, G., Palmer, C. a., Debattisti, V., Koshiba, T., Pulst, S.,
 457 Feldman, E.L., Hajnóczky, G., Shaw, J.M., 2014. Loss of Miro1-directed mitochondrial
 458 movement results in a novel murine model for neuron disease. *Proc. Natl. Acad. Sci.* 1–10.
 459 doi:10.1073/pnas.1402449111

460 Pinho, B.R., Santos, M.M., Fonseca-Silva, A., Valentão, P., Andrade, P.B., Oliveira, J.M.A.,
 461 2013. How mitochondrial dysfunction affects zebrafish development and cardiovascular
 462 function: an in vivo model for testing mitochondria-targeted drugs. *Br. J. Pharmacol.* 169,
 463 1072–90. doi:10.1111/bph.12186

464 Rahn, J.J., Stackley, K.D., Chan, S.S.L., 2013. Opa1 is required for proper mitochondrial
 465 metabolism in early development. *PLoS One* 8, e59218. doi:10.1371/journal.pone.0059218

466 Reis, K., Fransson, A., Aspenström, P., 2009. The Miro GTPases: at the heart of the
 467 mitochondrial transport machinery. *FEBS Lett.* 583, 1391–8.
 468 doi:10.1016/j.febslet.2009.04.015

469 Ro, H., Soun, K., Kim, E., Rhee, M., 2004. Novel Vector Systems Optimized for Injecting in
 470 vitro-synthesized mRNA into Zebrafish Embryos. *Mol. Cells* 17, 373–376.

471 Salas-Vidal, E., Meijer, A.H., Cheng, X., Spaink, H.P., 2005. Genomic annotation and
 472 expression analysis of the zebrafish Rho small GTPase family during development and
 473 bacterial infection. *Genomics* 86, 25–37. doi:10.1016/j.ygeno.2005.03.010

474 Saotome, M., Safiulina, D., Szabadkai, G., Das, S., Fransson, A., Aspenstrom, P., Rizzuto, R.,
 475 Hajnóczky, G., 2008. Bidirectional Ca²⁺-dependent control of mitochondrial dynamics by

476 the Miro GTPase. *Proc. Natl. Acad. Sci. U. S. A.* 105, 20728–33.
 477 doi:10.1073/pnas.0808953105

478 Sarraf, S. a, Raman, M., Guarani-Pereira, V., Sowa, M.E., Huttlin, E.L., Gygi, S.P., Harper,
 479 J.W., 2013. Landscape of the PARKIN-dependent ubiquitylome in response to
 480 mitochondrial depolarization. *Nature* 496, 372–6. doi:10.1038/nature12043

481 Saxton, W.M., Hollenbeck, P.J., 2012. The axonal transport of mitochondria. *J. Cell Sci.* 125,
 482 2095–104. doi:10.1242/jcs.053850

483 Schulte-Merker, S., Stainier, D.Y.R., 2014. Out with the old, in with the new: reassessing
 484 morpholino knockdowns in light of genome editing technology. *Development* 141, 3103–4.
 485 doi:10.1242/dev.112003

486 Sheng, Z.-H., 2014. Mitochondrial trafficking and anchoring in neurons: New insight and
 487 implications. *J. Cell Biol.* 204, 1087–98. doi:10.1083/jcb.201312123

488 Steele, S.L., Prykhodzhiy, S. V, Berman, J.N., 2014. Zebrafish as a model system for
 489 mitochondrial biology and diseases. *Transl. Res.* 163, 79–98. doi:10.1016/j.trsl.2013.08.008

490 Taylor, J.S., 2003. Genome Duplication, a Trait Shared by 22,000 Species of Ray-Finned Fish.
 491 *Genome Res.* 13, 382–390. doi:10.1101/gr.640303

492 Taylor, J.S., Van de Peer, Y., Braasch, I., Meyer, A., 2001. Comparative genomics provides
 493 evidence for an ancient genome duplication event in fish. *Philos. Trans. R. Soc. Lond. B.*
 494 *Biol. Sci.* 356, 1661–79. doi:10.1098/rstb.2001.0975

495 Thisse, B., Thisse, C., 2004. Fast Release Clones: A High Throughput Expression Analysis.
 496 ZFIN Direct Data Submiss. www.zfin.org.

497 Thisse, C., Thisse, B., 2008. High-resolution in situ hybridization to whole-mount zebrafish
 498 embryos. *Nat. Protoc.* 3, 59–69. doi:10.1038/nprot.2007.514

499 Tucker, B., Lardelli, M., 2007. A rapid apoptosis assay measuring relative acridine orange
 500 fluorescence in zebrafish embryos. *Zebrafish* 4, 113–116. doi:10.1089/zeb.2007.0508

501 Wager, K., Russell, C., 2013. Mitophagy and neurodegeneration: the zebrafish model system.
 502 *Autophagy* 9, 1693–709. doi:10.4161/auto.25082

503 Wang, X., Schwarz, T.L., 2009. The mechanism of Ca²⁺ -dependent regulation of kinesin-
 504 mediated mitochondrial motility. *Cell* 136, 163–74. doi:10.1016/j.cell.2008.11.046

505 Wang, X., Winter, D., Ashrafi, G., Schlehe, J., Wong, Y.L., Selkoe, D., Rice, S., Steen, J.,
 506 LaVoie, M.J., Schwarz, T.L., 2011. PINK1 and Parkin target Miro for phosphorylation and
 507 degradation to arrest mitochondrial motility. *Cell* 147, 893–906.
 508 doi:10.1016/j.cell.2011.10.018

509 Weihofen, A., Thomas, K.J., Ostaszewski, B.L., Cookson, M.R., Selkoe, D.J., 2009. Pink1 forms
 510 a multiprotein complex with Miro and Milton, linking Pink1 function to mitochondrial
 511 trafficking. *Biochemistry* 48, 2045–52. doi:10.1021/bi8019178

512 Weiser, D.C., Kimelman, D., 2012. Analysis of Cell Shape and Polarity During Zebrafish
 513 Gastrulation, in: *Planar Cell Polarity*. pp. 53–68. doi:10.1007/978-1-62703-239-1_1

514 Woods, I.G., Wilson, C., Friedlander, B., Chang, P., Reyes, D.K., Nix, R., Kelly, P.D., Chu, F.,
 515 Postlethwait, J.H., Talbot, W.S., 2005. The zebrafish gene map defines ancestral vertebrate
 516 chromosomes. *Genome Res.* 15, 1307–14. doi:10.1101/gr.4134305

517 Xi, Y., Ryan, J., Noble, S., Yu, M., Yilbas, A.E., Ekker, M., 2010. Impaired dopaminergic
 518 neuron development and locomotor function in zebrafish with loss of pink1 function. *Eur. J.*
 519 *Neurosci.* 31, 623–33. doi:10.1111/j.1460-9568.2010.07091.x

520 Yamaoka, S., Hara-Nishimura, I., 2014. The mitochondrial Ras-related GTPase Miro: views
 521 from inside and outside the metazoan kingdom. *Front. Plant Sci.* 5, 350.
 522 doi:10.3389/fpls.2014.00350

523 Yamaoka, S., Nakajima, M., Fujimoto, M., Tsutsumi, N., 2011. MIRO1 influences the
 524 morphology and intracellular distribution of mitochondria during embryonic cell division in
 525 Arabidopsis. *Plant Cell Rep.* 30, 239–44. doi:10.1007/s00299-010-0926-5

526

Figure Legends

Figure 1. The three zebrafish Rhot proteins are closely related and share conserved sites

with other Miro genes. A. Predicted GTPase and transmembrane (TM) domains are noted (Babic et al., 2015; Fransson et al., 2003, 2006). Essential glutamates within the two EF-hand domains necessary for calcium binding are indicated by E (Fransson et al., 2006). Proposed residues to form a salt bridge are indicated by S (Klosowiak et al., 2013). Putative Parkin ubiquitylation sites are indicated by U (Klosowiak et al., 2013; Sarraf et al., 2013), and a putative PINK1 phosphorylation site is indicated by P (Wang et al., 2011). B. A cladogram indicates the relationships among human (h) (**BC125104** and **BC014942**), mouse (m) (**NP_067511.4** and **NP_666111.1**), zebrafish (z), *Drosophila* (d) (**Q8IMX7.1**), and *Saccharomyces cerevisiae* (y) (**P39722.1**) proteins. Data were generated using Clustal W2 (McWilliam et al., 2013).

Figure 2. Like the hMiro proteins, Rhots localize to mitochondria when expressed in

mammalian tissue culture cells. A. Each hMiro or Rhot gene was tagged at the N-terminus with a myc tag and transfected into COS7 cells. Immunofluorescence was performed with a Tom40 antibody (middle) and a myc antibody (left) to determine that the proteins localize to the mitochondria (overlay, right). B. Rhots localize to the outer membrane of the mitochondrial fraction of transfected tissue culture cells. Myc-tagged Rhot constructs were transfected into HEK293T cells for protein expression and mitochondrial fractionation. Mitochondrial fractions were then treated with Proteinase K (100 µg/mL; Prot K) to digest exposed outer membrane proteins or treated with Triton X (0.1%; TX) and 20 µg/mL Proteinase K to solubilize mitochondria and digest all proteins. Untreated and treated fractions were resolved on 12% SDS-PAGE and immunoblotted for myc and Tom40.

Figure 3. Zebrafish Rhot2 and Rhot1b proteins interact with human Trak proteins in a yeast two hybrid assay. Each hMiro or Rhot gene was cloned in-frame with the binding domain of GAL4, and the two human Trak genes were cloned in-frame with the activation domain of GAL4. After mating, interactions are demonstrated on –ADE-HIS-LEU-TRP media supplemented with X- α -gal. The orientation of the plate is indicated at left. A summary of interactions is indicated in the table.

Figure 4. All three *rhots* are expressed throughout early development. A. PCRs amplifying the 3'UTR of each gene from whole embryo cDNA demonstrate expression at all stages examined. B. Shield stage embryos have ubiquitous expression of each *rhot*. C. Although *rhot2* demonstrates the strongest signal, all three genes have expression throughout the body in embryos at 1 dpf. D. 2dpf embryos.

Figure 5. Triple morphants demonstrate defects in axis elongation. Injection of a single MO at a low dose (0.75ng *rhot1a*, 4ng *rhot1b*, or 4ng *rhot2*, respectively) is not sufficient to produce a phenotype (left), but injection of all three MOs (3MO) at this dose yields a morphant phenotype (right). An uninjected embryo, top, is shown as a control.

Figure 6. Triple morphants have increased cell death by acridine orange staining. Uninjected or triple morphants (3MO) embryos at 1 dpf were stained with 2 μ g/mL acridine orange in E3 for 30 minutes, washed with E3, and imaged using a FITC filter.

Figure 1

A.

		GTPase I	
RHOT1A	1	MRKDVRIILLVGE	PKVGKTS
RHOT1B	1	MRKDVRIILLVGE	PKVGKTS
RHOT2	1	MRKDVRIILLVGE	PKVGKTS
RHOT1A	61	AEQSD	EQLVQ
RHOT1B	61	AEQSD	EQLVQ
RHOT2	61	NEQDDEV	REETV
RHOT1A	121	DLVEHSSMETILPIMNOYSEIETC	VECSAKNLKNI
RHOT1B	121	DLVEHSSMETILPIMNOYSEIETC	VECSAKNLKNI
RHOT2	121	DLRSGSSMETILPIMNOYSEIETC	VECSAKNLKNI
RHOT1A	181	MKPSCIKALTRIPKISDL	DNDGILNDNELNFFQ
RHOT1B	181	MRTACVLALTRIPKISDL	DNDGILNDNELNFFQ
RHOT2	181	LKAQCVRALSRIPKISDL	DNDGILNDNELNFFQ
RHOT1A	241	VKDNGLTILKGF	FLHTLF
RHOT1B	241	VKDNGLTILKGF	FLHTLF
RHOT2	241	VKDNGLTILKGF	FLHTLF
RHOT1A	301	LNNHAYLFLOS	VFDKHKDRD
RHOT1B	301	LNNHAYLFLOS	VFDKHKDRD
RHOT2	301	LNNHAYLFLOS	VFDKHKDRD
RHOT1A	361	GYLSQWTLT	TYLDVORCLEYL
RHOT1B	361	GYLSQWTLT	TYLDVORCLEYL
RHOT2	361	GYLSQWTLT	TYLDVORCLEYL
		GTPase II	
RHOT1A	421	CNVLGARGCGKSG	FLQAF
RHOT1B	421	CNVLGARGCGKSG	FLQAF
RHOT2	421	CKVIGPRCTGKT	DFLRAFLOR
RHOT1A	481	EFLSEADLACDV	CLVYDISN
RHOT1B	481	EFLSEADLACDV	CLVYDISN
RHOT2	480	EFLSEADLACDV	CLVYDISN
RHOT1A	541	LSPLDFCRKHKHL	HPQLETCNTTE
RHOT1B	541	MSPLDFCRKHKHL	HPQLETCNTTE
RHOT2	540	MSPLDFCRKHKHL	HPQLETCNTTE
RHOT1A	582	-----	HMTOADLKNS
RHOT1B	601	NFLNSELVQTV	RTKLYTVLSR
RHOT2	580	-----	HLNGSDMSST

B.

Figure 2**A.** **B.**

Figure 3

	vector only	hMiro1	hMiro2	Rhot1a	Rhot1b	Rhot2
vector only	-	-	-	-	-	-
hTrak1	-	+++	+++	-	+	+++
hTrak2	-	+++	++	-	+	++

A. **B.** **C.** **D.** **Fig. 4**

Figure 5

Figure 6