Signaling by Folded gastrulation is modulated by mitochondrial fusion and fission

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Summary
Mitochondria are increasingly being identified as integrators and regulators of cell signaling pathways. Folded gastrulation (Fog) is a secreted signaling molecule best known for its role in regulating cell shape change at the ventral furrow during gastrulation in Drosophila. Fog is thought to signal, through a G-protein-coupled receptor, to effect downstream cytoskeletal changes necessary for cell shape change. However, the mechanisms regulating Fog signaling that lead to change in cell morphology are poorly understood. This study describes the identification of proteins involved in mitochondrial fusion and fission as regulators of Fog signaling. Pro-fission factors were found to function as enhancers of signaling, whereas pro-fusion factors were found to have the opposite effect. Consistent with this, activation of Fog signaling resulted in mitochondrial fragmentation, and inhibiting this process could attenuate Fog signaling. The findings presented here show that mitochondria, through regulation of fusion and fission, function as downstream effectors and modulators of Fog signaling and Fog-dependent cell shape change.

Key words: Fog, Mitochondria, Signaling, Mitochondrial fusion, Mitochondrial fission

Introduction
Cell shape and regulation of cell shape change play an important role in the organization of tissues and organ structures. During development, the process of gastrulation is one of the earliest events that is dependent on cell shape change: cells undergo apical constriction in order to invaginate and form germ layers. In Drosophila, the signaling molecule Folded gastrulation (Fog) is a key zygotic factor required for coordinating cell shape change at the ventral furrow and posterior midgut primordium (PMG) during gastrulation (Costa et al., 1994). In the blastoderm embryo, the ventral-most cells, along with cells of the PMG, express fog. Activation of Fog signaling leads to apical flattening followed by constriction, facilitating invagination of epithelial cells through the ventral furrow and PMG to give rise to the mesoderm and posterior endoderm, respectively (Leptin 1999). In the absence of Fog, the process of cell shape change and cell invagination at the ventral furrow occurs in an uncoordinated manner, resulting in an irregular ventral furrow with a subsequent delay in the formation of the mesoderm. Genetic studies have shown that the genes concertina and RhoGEF2 function downstream of fog (Morize et al., 1998; Barrett et al., 1997; Häcker and Perrimon, 1998). Concertina encodes the alpha subunit of a heterotrimeric G-protein of the G12/13 family, and mutants in this gene exhibit defects at the ventral furrow that are similar to fog mutants (Parks and Wieschaus, 1991). Activation of Fog leads to the apical localization of myosin to facilitate apical constriction (Dawes-Hoang et al., 2005). Despite the identification of some of the downstream factors involved in mediating the Fog signal, the pathway leading to the apical localization of myosin and the regulation of subsequent apical constriction is not well understood. Post gastrulation, fog is expressed in a subset of longitudinal or interface glia in the embryonic central nervous system (CNS). Knockdown of fog, using RNA interference (RNAi), results in altered glial morphology thus affecting axonal ensheathment (Ratnaparkhi and Zinn, 2007).

Mitochondria are emerging as regulators of cell division and cell differentiation during development (Owusu-Ansah et al., 2008; Mitra et al., 2009; Nagaraj et al., 2012; Mitra et al., 2012). This study uncovers a role for the mitochondria as a novel and essential downstream mediator of Fog signaling. I demonstrate that Fog signaling leads to mitochondrial fission; inhibiting fission through downregulation of drp1 attenuates Fog signaling, whereas promoting fission by expression of UAS-drp1 or UAS-marf RNAi, enhances it. Furthur, I show that inhibiting mitochondrial fission, by knockdown of drp1 ventrally in blastoderm embryos, results in an irregular ventral furrow similar to fog mutants. Finally, I demonstrate that the presence of stable actin inhibits Fog-dependent mitochondrial fission. Taken together, these results suggest that mitochondrial fragmentation is an essential downstream event of Fog signaling and Fog mediated cell shape change.

Results
Genetic screen to identify modifiers of Fog signaling
Fog signaling is believed to be mediated by a seven transmembrane G-protein-coupled receptor (GPCR). The identity of this receptor is still unknown. To identify GPCR(s) and other modifiers that interact with Fog signaling, a targeted RNAi-based genetic screen was conducted using the wing as an assay system. Overexpression of Fog using MS1096-GAL4 (henceforth referred to as MS-GAL4) located on the X chromosome, resulted in a strong wing defect in all MS-GAL4; UAS-fog males with complete penetrance. The wings appeared short, sometimes stub like, and at
Fig. 1. Schematic representation of the genetic screen to identify interactors of fog. (A) A male of the genotype MS1096-GAL4/UAS-fog/CyoGFP. The wings are small and abnormal. (B) A flow chart describing the screen. Virgin females of the genotype MS1096-GAL4/FM7a; UAS-fog/CyoGFP were crossed to males from individual target RNAi lines. GAL4-positive males in the F1 were screened for their wing morphology. Lines that produced revertant males were scored as suppressors; those that produced males and females with wing defects were scored as enhancers; lines that produced wing defects in males but no significant defects in females were scored as non-interactors.

times necrotic (Fig. 1A). By contrast, wings in MS-GAL4; UAS-fog females appeared normal. However, increasing the dosage of fog by either increasing the copy number of the GAL4 or the UAS, led to wing defects similar to those observed in males (supplementary material Fig. S1). This difference in the phenotype between males and females was used in a screen to identify potential enhancers and suppressors of fog. Upon crossing MS-GAL4; UAS-fog females to males from individual UAS-RNAi lines, F1 males of the appropriate genotype were screened for their wing morphology. Wings of GAL4-positive F1 males were categorized into three classes: those with a complete or near complete wing blade were scored as ‘very good’ and ‘good’ rescue, respectively; those with partial wings, in most cases lacking the posterior compartment, or with just a single normal wing were considered as ‘partial rescue’. Males in which the wings resembled the parental male were scored as ‘poor rescue’. RNAi lines that rescued the wing phenotype in males but produced no significant phenotype in females were scored as ‘suppressors’; those that failed to rescue the wing phenotype in males and produced wing defects in females were scored as ‘enhancers’ (Fig. 1, and see Materials and Methods). As a proof-of-principle test, MS1096-GAL4/UAS-fog females were crossed to concertina (cta) and ptp52F mutant males because both genes are known to function downstream of fog (Morize et al., 1998; Ratnaparkhi and Zinn, 2007). As expected, in both cases, F1 males of the genotype MS1096-GAL4; UAS-fog/pta52F and MS1096-GAL4; UAS-fog/cta showed a strong suppression of the wing phenotype (Fig. 2B,C, respectively). Using this strategy, 222 RNAi lines representing primarily GPCRs and some signaling molecules were screened. A strong suppressor to emerge from the primary screen was CG8972 – the gene rhomboid-7 (rho-7, Fig. 2D). In the screen, crosses set up with two independent RNAi lines against CG8972 showed 74% and 66% suppression, respectively, of the wing phenotype (Fig. 2F). The suppression did not appear to be caused by a titration effect owing to the single GAL4 line driving expression of two transgenes because, in the screen, RNAi lines that failed to interact with fog, produced F1 males with wing phenotypes similar to parental MS1096-GAL4; UAS-fog males despite the presence of two transgenes in the background (UAS-fog and UAS-RNAi).

To validate this result, males carrying a loss-of-function mutation for rho-7 were crossed to MS1096-GAL4; UAS-fog females. Approximately 43.5% of MS1096-GAL4; UAS-fog/ARho-7 males, showed rescue of the wing phenotype whereas 37% showed only a partial rescue (Fig. 2E,F) indicating that Rho-7 is indeed a suppressor of Fog signaling.

Fog signaling interacts with regulators of mitochondrial fusion and fission

Rhomboid-7 (Rho-7) is a highly conserved mitochondrial intramembrane protease belonging to the Rhomboid family of serine proteases. Rho-7 localizes to the inner mitochondrial membrane and is thought to regulate mitochondrial fusion in Drosophila (McQuibban et al., 2006). Given the interaction between fog and rho-7, genes that constitute the core of mitochondrial fusion and fission machinery were screened for their interaction with fog. Dynamin Related Protein 1 (drp1), a GTPase belonging to the dynamin family, is a key regulator of mitochondrial fusion (Verstreken et al., 2005). Loss of drp1 results in increased fusion leading to formation of long filamentous mitochondria. Interestingly, expression of drp1 dsRNA using two independent RNAi lines resulted in a 77% and 83% suppression of the wing phenotype, suggesting that loss of mitochondrial fission inhibits fog signaling (Fig. 3A,E). Consistent with this result, overexpression of drp1 enhanced the wing phenotype with complete penetrance: the wing phenotype of MS1096-GAL4; UAS-fog, UAS-drp1 was similar to parental MS1096-GAL4 > UAS-fog males, with the corresponding females showing strong wing defects ranging from relatively mild margin defects to strong wing defects that were similar to those in males (Fig. 3C,E). Among the progeny of the same cross, animals carrying one copy of the GAL4 and UAS-drp1, but not UAS-fog (MS1096-GAL4 > UAS-drp1, CyoGFP) showed normal curly wings indicating that the observed enhancement was indeed because of an interaction between fog and drp1, and not due an independent phenotype caused by overexpression of drp1. This was also confirmed independently by crossing UAS-drp1 to MS1096-GAL4 females. Overexpression of drp1 did not produce any wing defect.

As with fission, the process of mitochondrial fusion is also regulated by GTPases. In yeast, Fzo1 and Mgm1 constitute the core machinery regulating fusion of the outer and inner mitochondrial membrane respectively (Hermann et al., 1998; Wong et al., 2003; Meeusen et al., 2006). Mammalian systems
Fig. 2. Downregulation of rhomboid-7 suppresses the wing phenotype caused by fog overexpression. (A) Male animal of the genotype MS1096-GAL4; UAS-fog+. The animals show a drastic loss of the wing blade, which is sometimes necrotic. Removal of one copy of pps2F18.3 (B) or ctaRC10 (C), which function downstream of fog, suppress the wing phenotype. (D) Knockdown of CG8972 (rhomboid-7) using RNAi leads to a strong suppression of the wing phenotype. (E) Loss of one copy of rho-7 rescues the wing phenotype. (F) Graph showing percentage rescue observed in each cross. Black indicates poor or no rescue; light gray represents rescue of the wing phenotype to normal or near normal morphology; dark gray bars indicate partial rescue of the wing phenotype. Control MS1096-GAL4×UAS-fog males exhibit ‘poor’ wings with complete penetrance. This phenotype is completely suppressed by loss of one copy of either cta or pps2F. Expression of dsRNA against rho-7, using the two independent lines CG8972i-1 and CG8972i-2, leads to 74% and 66% suppression of the wing phenotype, respectively. In animals that lack a copy of rho-7 (ΔRho7), ~43% show rescued wings; 37% show partial rescue, and the remaining 20% fail to show any rescue of the wing phenotype.

Fig. 3. Fog signaling is modulated by mitochondrial fusion and fission. (A) Knockdown of drp1 using RNAi, suppresses the wing phenotype in MS1096-GAL4; UAS-fog males. (B,B′) Downregulation of marf enhances the effect of fog overexpression: both males (B) and females (B′) show strong wing defects. (C,C′) Overexpression of drp1 also enhances Fog signaling. Similar to control males shown in Fig. 2A, the wings appear small and stub-like in males (C) and wing defects are seen in all females, with a majority showing loss of the posterior compartment (C′, double arrow). (D) Expression of marf rescues the wing phenotype caused by Fog overexpression. (E) Graph representing the percentage rescue observed in each cross: light gray bars represent ‘rescue’ with wing blades having a normal or near normal morphology; black bars represent poor or no rescue; dark gray bars represent partial rescue. Expression of drp1 dsRNA using two independent RNAi lines (drp1-1) and (drp1-2), leads to 77% and 83% rescue of the wing phenotype, respectively; downregulation of marf or overexpression of drp1 does not rescue the wing phenotype. Expression of marf results in a strong suppression of the wing phenotype with 81% of the males showing normal wing morphology.
have two functional homologs of Fzo1: mitofusin1 (MFN1) and mitofusin2 (MFN2). In *Drosophila*, the genes *fuzzy onions* (*fzo*) and *Mitochondrial assembly regulatory factor* (*marf*) encode homologs of mitofusin. However, *fzo* is expressed exclusively in the male germline, whereas *marf* is expressed extensively in both somatic and germ tissue (Hwa et al., 2002). Consistent with its role as a fusion-promoting factor, downregulation of *marf* is known to promote mitochondrial fragmentation (Deng et al., 2008). On the basis of the results with *drp1*, it was hypothesized that promoting mitochondrial fission should enhance Fog signaling. To test this, dsRNA against *marf* was expressed using UAS-*Marf* dsRNA (Deng et al., 2008). Knockdown of *marf* using this RNAi line led to strong pupal lethality making it difficult to assess its interaction with *fog*. However, expression of *marf* dsRNA using the weaker RNAi line (P'[TRIP: JF01650]; attP2) in *MS1096-GAL4>UAS-fog* animals, led to a clear enhancement of the wing phenotype with complete penetrance: in males, the wings appeared small or stub-like, similar to parental males, and all females showed short and abnormal wings, indicating that inhibiting mitochondrial fusion through downregulation of *marf* potentiates Fog signaling (Fig. 3B,B'). Similar to *UAS-drp1*, control flies in the same cross carrying a single copy each of the GAL4 and *UAS-marf* dsRNA showed no wing defect, thus ruling out the possibility of any additive effect caused by the RNAi. Further supporting this, expression of *marf* suppressed the wing phenotype of *MS1096-GAL4; UAS-fog* males: in more than 80% of F1 males, the wings appeared normal, whereas the F1 females of the corresponding genotype did not show any wing defect (Fig. 3D,E). These results thus clearly show that Fog signaling can be modulated by mitochondrial fusion and fission events.

**Overexpression of ***fog** leads to mitochondrial fragmentation in a *drp1*-dependent manner**

The interaction between *fog*, *drp1* and *marf* suggests that mitochondrial fragmentation is an event downstream of Fog signaling. To test this, Fog was expressed in third-instar larval muscles using *MHC-GAL4* and mitochondrial morphology was visualized through co-expression of UAS-mitoGFP. In control animals of the genotype UAS-mitoGFP/++; *MHC-GAL4/+* (Mhc-), the mitochondria were seen present as a dense network around nuclei (Fig. 4A,A). However, in animals overexpressing *fog*, the mitochondrial appearance small and spherical or elliptical in shape, indicating fragmentation (Fig. 4B,B'). This effect was observed in animals raised at both 25°C and 28°C. To determine whether the effect on mitochondria was due to Fog alone or the signaling pathway activated by Fog, a constitutively active form of Concertina (UAS-cta<sup>Q303E</sup>) was expressed in muscles. As for Fog, expression of Cta<sup>Q303E</sup> resulted in mitochondria that were small and spherical, indicating fragmentation (Fig. 4C,C'). These results thus seemed to suggest that activation of the pathway, and not Fog, leads to mitochondrial fragmentation. To test whether this process was *drp1* dependent, dsRNA against *drp1* was expressed in *MHC-GAL4>UAS-mito, UAS-fog* animals. Consistent with the observed genetic interaction between *fog* and *drp1*, the muscles of these animals showed long and tubular mitochondria, similar to those in the control (Fig. 4D,D'). This effect did not seem to be caused by reduced expression of Fog owing to a dilution effect of the GAL4 because, in animals expressing GFP instead of *drp1* dsRNA, the mitochondria appeared fragmented as expected (supplementary material Fig. S2).

**Fig. 4. Fog-mediated mitochondrial fission is dependent on Drp1.** (A–D) Images of third-instar larval muscles. (A'–D') Corresponding enlarged versions of the images shown in A–D. (A,A') Shown is the dense mitochondrial network in a third-instar larval muscle of a control animal (*Mhc-GAL4>UAS-mito::GFP*) stained with anti-GFP antibody. (B,B') In animals expressing *UAS-fog*, mitochondria are fragmented and appear as small puncta. (C,C') Expression of a constitutively active form of concertina (*cta<sup>Q303E</sup>*) also causes mitochondrial fragmentation. Similar to animals expressing *UAS-fog*, the mitochondria appear small and spherical. (D,D') Downregulation of *drp1* in animals expressing *UAS-fog* inhibits mitochondrial fission. The mitochondrial network appears more filamentous. Scale bar: 10 μm.

**Loss of Fog leads to mitochondrial fusion**

Activation of Fog signaling leads to mitochondrial fragmentation. It was therefore of interest to determine the effect of *fog* loss-of-function on mitochondrial morphology. Interestingly, knockdown of *fog* through expression of *fog* dsRNA resulted in multiple mitochondrial morphologies. Most frequently, the mitochondria appeared predominantly long and filamentous (27.58%, *n* = 30) or small and fragmented (24.13%, *n* = 30) which, at first glance, appeared to be similar to animals expressing UAS-*fog* (Fig. 4B). However, on closer examination, these mitochondria were found to be ‘donut’ shaped (Fig. 5A,A', arrows). Donut-shaped mitochondria are thought to represent autofused forms of mitochondria. These are thought to arise as breakaway products of long filamentous mitochondria which form because of increased fusion driven either by overexpression of pro-fusion factors or loss of fission factors (Cui et al., 2010). A relatively less common phenotype was the formation of large amorphous aggregates especially around nuclei (20.69%; Fig. 5B,B'). Similar ‘hyperfused’ mitochondria have been observed in cells during the G1-S transition of the cell cycle (Mitra et al., 2009). In about 27% of the muscles, mitochondria showed mixed morphologies consisting of elongated as well donut-shaped forms. The association of each of these different conformations with
excessive fusion suggests that loss of fog promotes mitochondrial fusion. To further confirm this, fog knockdown was carried out using another independent RNAi line (UAS-fog dsRNA). Knockdown of fog using this RNAi line led to formation of highly filamentous mitochondria (Fig. 5C). Taken together, these results suggest that loss of fog promotes mitochondrial fusion.

Knockdown of drp1 leads to ventral furrow defects

The above results show that activation of Fog signaling leads to mitochondrial fragmentation and, inhibiting this process can attenuate Fog signaling. It was therefore hypothesized that inhibiting mitochondrial fission in cells that are responsive to Fog signaling should phenocopy fog loss-of-function. The earliest known function of fog is at the ventral furrow, where it is known to elicit cell shape changes essential for coordinated invagination of the presumptive mesoderm. Loss of Fog leads to asynchronous invagination of cells resulting in the formation of an irregular furrow (Costa et al., 1994). To test whether inhibiting mitochondrial fission in blastoderm embryos results in asynchronous or delayed invagination of cells at the ventral furrow, transgenic dsRNA against drp1 was expressed in blastoderm embryos using fog-GAL4. The expression of this GAL4 is detected ventrally in embryos at the time of cellularization (Fig. 6A). Embryos between 0 and 3 hours were collected from the cross at 25°C and stained with an antibody against Twist, a transcription factor expressed in cells of the presumptive mesoderm (Thisse et al., 1988). In most embryos, the ventral furrow appeared as a sharp, narrow and straight indentation along the anterior-posterior axis on the ventral side (Fig. 6B, black arrow). The ‘T-bar’ present in the anterior part of the ventral furrow, through which the primary head mesoderm (PHM) invaginates, was well formed (Fig. 6B, white arrow) (de Valasco et al., 2006). However, in a quarter of the embryos (n=20), the ventral furrow appeared as an irregular shallow line. Twist-positive cells were seen at the surface in the region of the ventral furrow indicating asynchronous and/or delayed invagination (Fig. 6D, asterisk). More commonly, the T-bar failed to form a ‘ring’ as it does in wild-type embryos (Fig. 6D, asterisk) and Twist staining towards the posterior end appeared broad (Fig. 6D, arrowhead). Thus, loss of drp1 produced defects at the ventral furrow cells in a manner similar to fog.

Destabilized F-actin enhances Fog signaling

Regulation of mitochondrial dynamics in response to cell signaling is known to occur through modulation of expression, activity or localization of proteins that constitute the fusion–fission machinery (Rambold et al., 2011; Nagaraj et al., 2012; De Vos et al., 2005). With respect to the latter, actin has been shown...
to be involved in localization of Drp1 to the mitochondria, thereby regulating fission. Fog signaling leads to apical constriction through reorganization of the actomyosin network. Given that the end point of Fog signaling is remodeling of actin cytoskeleton, it seemed relevant to ask whether actin plays a role in regulating Fog-mediated mitochondrial fragmentation. To explore this possibility, actin-regulating proteins like Cofilin and Gelsolin were expressed in MS1096-GAL4, UAS-fog animals. Cofilin and Gelsolin are actin-severing proteins involved in the breakdown of F-actin. Downregulation of Cofilin through expression of transgenic dsRNA led to strong larval and pupal lethality. However, expression of gelsolin enhanced Fog signaling, resulting in abnormal wing morphologies in both males and females (Fig. 7A,B). Expression of gelsolin alone did not give rise to any wing defect, indicating that the observed enhancement was due to its interaction with fog. To test whether the converse was also true, WASP, an actin nucleator that promotes synthesis of F-actin, was expressed in MS1096-GAL4, UAS-fog animals. Suppression of the wing phenotype was observed in about 53.3% of the animals (Fig. 7C,D). Interestingly, co-expression of UAS-WASP and UAS-fog in MHC-GAL4>UAS-mitoGFP animals, resulted in long filamentous mitochondria as opposed to the fragmented forms associated with UAS-fog. To ensure that Fog was indeed expressed in these animals, antibody staining was carried out using anti-Fog and anti-GFP antibodies. In control animals, the mitochondria appeared as a network (Fig. 7E) and Fog expression was present uniformly in muscles (Fig. 7F). In animals expressing both transgenes, punctate Fog expression was observed all over the muscle (Fig. 7I). However, the filamentous mitochondrial morphology was observed in these animals (Fig. 7H,J). This indicates that an increase in stable F-actin due to WASP expression can suppress Fog-mediated mitochondrial fragmentation and that actin functions downstream of Fog to mediate mitochondrial fission.

**Discussion**

Fog is a secreted signaling molecule known to regulate apical constriction of cells at the ventral furrow during gastrulation in *Drosophila*. In this study, I describe a genetic screen conducted to identify regulators of Fog signaling using the adult wing as an assay system. Fog is expressed in the wing disc; heterozygous mutant combinations of fog, cta and RhoGEF2, result in deformed wings (Nikolaaidou and Barrett, 2004). Given a functional role for Fog in wing development, it seemed relevant to screen for interactors using the wing as an assay system. Through the identification of proteins involved in mitochondrial fusion and fission as regulators of Fog signaling, I show, for the first time, a role for the mitochondria as downstream effectors of Fog signaling.

Overexpression of Fog leads to mitochondrial fission. A similar phenotype was observed upon expression of a constitutively active form of concertina suggesting that this effect is a consequence of the signaling pathway and not Fog expression was present uniformly in muscles (Fig. 7F).
protein per se (Fig. 4). Conversely, knockdown of Fog results in different mitochondrial morphologies, all of which are known to be associated with excessive or enhanced fusion. In the present study, expression of fog dsRNA led to formation of highly filamentous, donut-shaped or fused amorphous forms of mitochondria (Cui et al., 2010; Mitra et al., 2009). Whether these different morphologies arise due to differences in the extent of fog knockdown is not clear at this point.

Fog-mediated mitochondrial fission is dependent on Drp1. Inhibiting fission through downregulation of drp1 suppresses Fog signaling (Fig. 3). Consistent with this, knockdown of drp1 in cells that respond to Fog in the blastoderm leads to invagination defects at the ventral furrow (Fig. 6). Interestingly, in a study by Gong and colleagues, drp1 was identified as a differentially expressed factor at the ventral furrow. Injection of dsRNA against drp1 was found to result in ventral furrow defects ranging from mild to severe. (Gong et al., 2004). In the experiments described here, the ventral furrow phenotype due to transgenic knockdown of drp1 were not as severe as those observed in fog mutants; one reason for this could be insufficient expression of drp1 dsRNA. Relatively stronger effects were seen in embryos in which expression of the RNAi was carried out at 28°C. Some of these phenotypes were also observed in drp1-KO03815 mutants (data not shown). However, the PMG phenotype associated with fog loss-of-function was not seen in these mutants. One reason for this could be the presence of maternal drp1 mRNA that compensates for the loss of any zygotic drp1 expression. Nonetheless, the observation that inhibiting mitochondrial fission results in a fog loss-of-function-like phenotype correlates well with observed interaction between fog and drp1 and suggests that mitochondrial fission is necessary for coordinated cell shape change at the ventral furrow.

The differential effect of fog gain-of-function and loss-of-function on mitochondrial morphology also indicates that the system might be used by the cell to ‘sense’ and distinguish one scenario from the other, pointing towards a very sensitive role for mitochondria in regulating Fog signaling. In this context, it would be interesting to test whether the production of reactive oxygen species (ROS) or any other ‘mitochondrial output’ is altered in response to Fog signaling because this could be an additional parameter the cell could use to respond appropriately to Fog. It would also be interesting to see whether the involvement of mitochondria in regulation of cell shape change is more widespread than previously known and whether the effect of Fog on mitochondrial morphology is context dependent. These will need to be tested more rigorously.

Generation of cell asymmetry and its regulation by mitochondrial fission and fusion pathways has been previously observed in migrating lymphocytes. In these cells, activation of G-protein signaling in response to chemokines was found to result in the redistribution and accumulation of mitochondria in the uropod or non migrating front to provide the ATP essential for actomyosin contraction during migration. The process of redistribution was shown to be dependent on mitochondrial fission such that inhibiting fission led to a loss of cell polarization and inhibition of migration (Campello et al., 2006). More recently, in a study by Muyalil and colleagues (Muliyl et al., 2011), mitochondrial fission mediated by drp1 was found to be necessary and sufficient for delamination of cells of the amnioserosa during dorsal closure, a process that occurs through actomyosin contraction. Although these studies, together with the one described here, suggest a wider role for mitochondria in regulating actomyosin-based contraction and cell shape change, fragmentation of mitochondria in response to Fog might be required for relocation of mitochondria to the site of action to provide the necessary energy for apical constriction.

How might Fog regulate mitochondrial fragmentation? One possibility is that activation of Fog signaling modulates expression or localization of drp1 in a manner that promotes mitochondrial fission. Drp1 is usually a cytoplasmic protein that gets recruited to the mitochondria during fission. Many studies on mammalian Drp1 (Dynamin-related protein 1) have shown the recruitment to be regulated by post-translational modifications such as phosphorylation and sumoylation (Santel and Frank, 2008). However, on the basis of the interaction between Fog and actin regulators it is likely that actin might be involved in mediating fission. Recent studies have shown that actin can regulate recruitment of Drp1 to mitochondria in a context-dependent manner and thus control the process of fission (De Vos et al., 2005; DuBoff et al., 2012). In a study by DuBoff and colleagues, excessive stable F-actin was shown to lead to the accumulation of Drp1 and prevent it from localizing to mitochondria in a process dependent on the non-muscle Myosin II, resulting in long elongated mitochondria (DuBoff et al., 2012). The suppression of Fog-mediated mitochondrial fission by the expression of WASP, an actin nucleator, suggests that a similar mechanism could also be operating in this context.

This study was initiated through the identification of rhomboid-7 as a suppressor of fog. At this point, it is unclear how Rho-7, a pro-fusion factor, might interact with Fog signaling. It is likely that the interaction is independent of its role in mitochondrial fusion. This will need further investigation.

Materials and Methods

Drosophila stocks and genetics

All fly stocks were raised on standard cornmeal medium. In the screen, MS1096-GAL4/FM7a; UAS-fog/CyO;GFP females were crossed to UAS-dsRNA males. Non-balancer F1 males were screened for their wing phenotype. Screening of RNAi lines, comprising 111 GPCR genes, was carried out in the Fly facilities at NCBS, Bangalore, and JINER, Pune, using lines generated by Vienna Drosophila RNAi Center (VDRC) (Dietz et al., 2007) and National Institute of Genetics, Japan. The list of Drosophila GPCRs used in the screen was generated primarily using data from published literature (Brody and Cravchick, 2000; Hauser et al., 2006; Banant et al., 2005) and BLAST searches against the Drosophila genome using a few representative GPCRs. Transgenic RNAi lines CG89721-1 (CG89721-1), CG89721-2 (CG89721-2), drpRNAi-1(CG89721-1), and drpRNAi-2 (CG3210-1676) were from the VDRC stock centre. UAS-drp1 (Deng et al., 2008); ARho-7 and UAS-Rho-7 (McQuibban et al., 2006); UAS-gelcollin and UAS-marf (DuBoff et al., 2012) are as described previously. UAS-cyO3126; (kind gift of Naoyuki Fuse), UAS-marf RNAs (P[TripF01650];attP2) and UAS-mito;GFp, were from the Bloomington Drosophila Stock Centre. The UAS-fog;M5, and UAS-fog;dsRNA55 used in this study have been described previously (Ratnaparkhi and Zinn, 2007). UAS-fog;dsRNA1-4 (Zinn laboratory, Caltech) was generated by cloning a region of the fog cDNA corresponding to amino acids 151–321 into the pWIZ vector. All crosses were carried out at 25°C.

Molecular biology and immunohistochemistry

fog;GAL4 was generated by amplifying a 1.1 kb genomic region immediately 5′ upstream of the transcription start site of fog by PCR, which was then sequenced and cloned into plasmid pFtGAL4. Transgenic flies were generated by the fly transgenic facility at Center for Cellular and Molecular Platforms (C-CAMP), Bangalore, India. (A detailed characterization of the GAL4 will be presented elsewhere.)

Immunostaining of whole-mount embryos and larval fillets was performed using standard protocols (Patel, 1994). Embryos and larvae of the correct genotype were identified using GFP balancers. In the case of larvae, animals of the correct genotype were sorted under a GFP stereomicroscope prior to dissection. The following antibodies were used: chicken anti-GFP (1:500, Life Technologies) and rabbit anti-twist (1:5000, S. Roth). Imaging was performed using the Zeiss 710 inverted confocal microscope and Zeiss Axiovert.
epifluorescence microscope at ISER, Pune. Images of adult flies were taken on a Leica microsystems S5APO light microscope. ImageJ (NIH, Bethesda) software was used for image analysis. The figures were assembled using Photoshop CS3 software and PowerPoint.

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References


**Fig. S1. Fog overexpression using MS1096-GAL4 in females is dose dependent.**  (A) A female fly of the genotype *MS1096-GAL4/FM7a; UAS-fog/CyoGFP*. These flies carry a single copy of the GAL4 and have curly wings.  (B) A female fly *MS1096-GAL4/MS1096-GAL4; UAS-fog/CyoGFP*. Presence of two copies of the GAL4 causes increased expression of *fog* resulting in short small wings which are sometimes necrotic (arrow).

**Fig. S2. Co-expression of UAS-mCD8GFP with UAS-fog does not alter fog mediated mitochondrial fragmentation.**  (A-C) Shown is a muscle of a MHC-GAL4> UAS-mitoGFP, UAS-mCD8GFP 3rd instar larva stained with anti-GFP (A, green) and anti-fog (B, red). Mitochondria appear as a network (arrows in A).  (D-F) Muscle of a 3rd instar larva of the genotype: *MHC-GAL4>UAS-mitoGFP, UAS-fog, UAS-mCD8GFP*. The mitochondria are fragmented (arrows, D); Fog expression is seen as puncta all over the muscle (E).