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Control of Myoblast Fusion by a Guanine Nucleotide Exchange Factor, Loner, and Its Effector ARF6

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Summary

Myoblast fusion is essential for the formation and regeneration of skeletal muscle. In a genetic screen for regulators of muscle development in *Drosophila*, we discovered a gene encoding a guanine nucleotide exchange factor, called *loner*, which is required for myoblast fusion. Loner localizes to subcellular sites of fusion and acts downstream of cell surface fusion receptors by recruiting the small GTPase ARF6 and stimulating guanine nucleotide exchange. Accordingly, a dominant-negative ARF6 disrupts myoblast fusion in *Drosophila* embryos and in mammalian myoblasts in culture, mimicking the fusion defects caused by loss of Loner. Loner and ARF6, which also control the proper membrane localization of another small GTPase, Rac, are key components of a cellular apparatus required for myoblast fusion and muscle development. In muscle cells, this fusigenic mechanism is coupled to fusion receptors; in other fusion-competent cell types it may be triggered by different upstream signals.

Introduction

Intercellular fusion is fundamental to the formation of multicellular organisms and is required for processes as diverse as fertilization, bone and placental development, and myogenesis (Hernandez et al., 1996; Blumenthal et al., 2003). Recent studies have also revealed a role for intercellular fusion in tissue repair by circulating hematopoietic stem cells (Blau, 2002). Whereas the mechanisms involved in intracellular membrane fusion are understood in considerable detail, there is a dearth of information on the molecules and mechanisms that govern cell-cell fusion. Understanding this process may provide opportunities for its manipulation, which has obvious and important implications for tissue engineering and repair.

During skeletal muscle development and regeneration following injury, mononucleated myoblasts fuse to form multinucleated muscle fibers. The process of myoblast fusion is amenable to genetic dissection in the fruit fly *Drosophila melanogaster*, in which muscle formation involves a well-defined temporo-spatial sequence of events that are remarkably conserved in mammalian myogenesis (Wakelam, 1985; Knudsen, 1992; Doberstein et al., 1997). Given the evolutionary conservation of the cellular and molecular events of muscle development, regulators of myoblast fusion identified genetically in *Drosophila* are likely to provide insights into mammalian myogenesis, as well as intercellular fusion in general.

The somatic musculature of the *Drosophila* embryo is derived from the embryonic mesoderm. During mid-embryogenesis, mesodermal cells expressing *twist* (*twi*) acquire a myoblast cell fate. Subsequently, a subset of myoblasts expressing *lethal of scute* is selected via lateral inhibition to become muscle founder cells, while the remaining *twi*-expressing cells become fusion-competent (Baylies et al., 1998; Frasch, 1999). The founder cells are a source of attractant(s) for surrounding fusion-competent myoblasts, and fusion between these two populations of cells leads to the formation of myotubes that incorporate between 4 and 25 myoblasts. The subsequent epidermal attachment of myotubes results in a highly stereotyped, segmentally repeated pattern of 30 muscle fibers per hemisegment (Bate, 1993).

Myoblast fusion is a multistep process involving the initial recognition and adhesion between muscle founder cells and fusion-competent myoblasts, subsequent alignment of two adhering cells, and ultimate membrane breakdown and fusion (Doberstein et al., 1997). Similar ultrastructural changes associated with these events occur in vertebrate and *Drosophila* muscle cells, as well as in nonmuscle cells that undergo fusion (Wakelam, 1985; Knudsen, 1992; Hernandez et al., 1996; Blumenthal et al., 2003). Genetic studies in *Drosophila* have begun to identify components of a possible signaling cascade required for myoblast fusion (Figure 7, for reviews see Paululat et al., 1999; Frasch and Leptin, 2000; Baylies and Michelson, 2001; Dworak and Sink, 2002; Taylor, 2002). *dumbfounded* (*duf*)/*kin of Irregular-chiasm-C* (*kirre*) and *roughest* (*rst*)/*irregular-chiasm-C* (*irreC*) encode paralogues of immunoglobulin (Ig) domain-containing transmembrane receptor-like proteins that are specifically required in founder cells (Ruiz-Gomez et al., 2000; Strunkelnberg et al., 2001). *sticks and stones* (*sns*) and *hibris* (*hbs*) encode two paralogues of transmembrane proteins with Ig domains that are expressed, and in the case of *sns* required, in fusion-competent myoblasts (Bour et al., 2000; Artero et al., 2001; Dworak et al., 2001). It has been suggested that DUF/KIRRE and RST/IRREC act as attractants for fusion-competent myoblasts by interacting with SNS and HBS (Ruiz-Gomez et al., 2000; Dworak et al., 2001; Strunkelnberg et al., 2001). The *antisocial* (*ants*)/*rolling pebbles* (*rols7*) gene encodes a founder cell-specific intracellular adaptor protein, which transduces fusion signals by linking membrane fusion receptors and the cytoskeleton (Chen and Olson, 2001; Menon and Chia, 2001; Rau et al., 2001). *myoblast city* (*mbc*) encodes a cytoskeleton-associated protein with homology to the human protein DOCK180 (Erickson et al., 1997). Recently, a DOCK180-ELMO complex was shown to function as a two-part unconventional guanine nucleotide exchange factor (GEF) for the small G protein Rac in phagocytosis (Brugnera et al., 2002). Interestingly, the *Drosophila* homologs of Rac, Drac1, and Drac2, play essential roles in myoblast fusion (Hakeda-Suzuki et al., 2002). Despite

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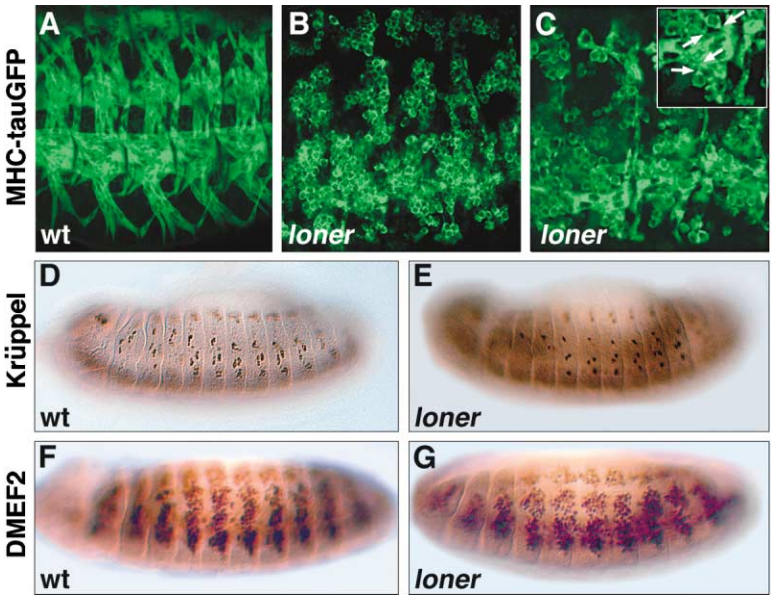

Figure 1. Myoblast Fusion Defect in *loner* Mutant Embryos

The somatic musculature in wild-type (A) and *loner*^{T1032} embryos (B and C) are visualized by a *MHC-tauGFP* reporter. Embryos are oriented with dorsal up and anterior to the left in this and all other figures.

(A) Ventrolateral view of a portion of a stage 14 wild-type embryo showing the segmentally repeated pattern of its somatic musculature.

(B and C) Lateral view of a portion of a stage 13 (B) and stage 14 (C) *loner*^{T1032} embryo in which myoblasts fail to fuse. Fusion-competent myoblasts extend filopodia (arrows) toward elongated founder cells, suggesting that adhesion between fusion-competent myoblasts and founder cells is not affected.

Wild-type (D and F) and *loner*^{T1032} mutant embryos (E and G) were stained with anti-KR (D and E) and anti-DMEF2 (F and G) antibodies.

(D and E) Lateral view of stage 13 wild-type and *loner*^{T1032} embryos stained for KR. In wild-type embryos, KR is initially expressed in a subset of founder cells, but is later turned on in other nuclei of the multinucleated fibers as KR-positive founder cells fuse to neighboring myoblasts. Thus, KR staining appears as clusters in the wild-type embryo (D). In the *loner* mutant embryo (E), KR is expressed in isolated, instead of clusters of, nuclei due to lack of fusion.

(F and G) Lateral view of stage 14 embryos showing similar number of DMEF2-expressing myoblasts in wild-type (F) and *loner*^{T1032} mutant (G) embryos.

the discovery of the DUF/RST→ANTS→MBC→Rac signaling pathway within founder cells, the complexity of the fusion process predicts additional molecules that function together with these components to accomplish the events of fusion.

Here, we describe the discovery and mechanisms of action of Loner, a GEF of the Sec7 family that acts downstream of myoblast fusion receptors. Loner, which is localized to subcellular sites of fusion, controls myoblast fusion by recruiting the small GTPase ARF6 and promoting its guanine nucleotide exchange. The Loner/ARF6 module acts in parallel to, and converges with, the ANTS→MBC→Rac pathway. This fusigenic mechanism, which is activated by specific fusion receptors in muscle cells, has the potential to control fusion of other cell types by coupling to different upstream effectors.

Results

Identification of *loner*

We performed a genetic screen in *Drosophila* using an *MHC-tauGFP* line to identify new genes involved in skeletal muscle development (E.H.C. and E.N.O., unpublished data). One complementation group on the third chromosome containing two EMS mutant alleles, *T1032* and *T1057*, showed a striking mutant phenotype in which the developing somatic muscle cells failed to fuse. Instead of mature, multinucleated muscle fibers, a large number of unfused myosin-expressing myocytes were present in mutant embryos (compare Figures 1A with 1B and 1C). Based on the failure of mutant myoblasts to fuse with surrounding cells, we named this locus *loner*. All skeletal muscles appeared to be affected in *loner* mutant embryos. In contrast, there were no gross defects in visceral muscles or the dorsal vessel (data not shown). The phenotype resulting from the *loner* mutation is therefore highly specific to the somatic musculature.

loner Is Specifically Required for Myoblast Fusion

In order to determine if the *loner* phenotype was due to a specific defect in myoblast fusion or secondary defects in myoblast fate determination or other developmental processes, we examined several developmental processes that might indirectly affect muscle differentiation, including the specification of muscle founder cells and myoblasts, the pattern of innervation by motor neurons, and differentiation of the epidermis. Muscle founder cell specification was assessed by expression of *Krüppel* (*Kr*), which is initially expressed in a subset of founder cell nuclei in wild-type embryos (Ruiz-Gomez et al., 1997). Later, as neighboring myoblasts fuse with KR-expressing founder cells, their nuclei also express KR, resulting in clusters of KR-positive cells in the embryo (Figure 1D). As shown in Figure 1E, KR was expressed in its characteristic positions in *loner* mutant embryos, suggesting that founder cell fate was properly specified. However, only one nucleus was present in each "cluster" of KR-expressing cells, suggesting that founder cells failed to fuse with surrounding fusion-competent myoblasts. We also examined *Dmef2*, which is expressed in the nuclei of all somatic, visceral, and cardiac myoblasts (Lilly et al., 1994; Nguyen et al., 1994) (Figure 1F). Wild-type and *loner* mutant embryos had comparable numbers of DMEF2-expressing cells, suggesting that the mutant myoblasts were properly specified despite a block in myoblast fusion (Figures 1F and 1G). In addition, antibody staining with fasciclin II (Grenningloh et al., 1991) revealed a normal pattern of muscle innervation by motor neurons and cuticle preparations

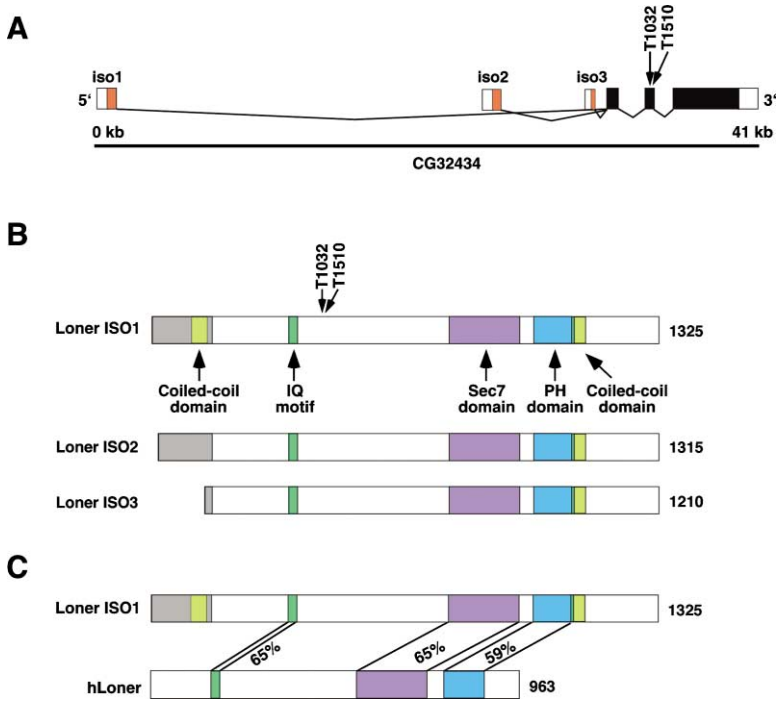

Figure 2. Molecular Characterization of *loner*

(A) Genomic organization of the *loner* gene. Three alternatively spliced forms are shown. Black boxes represent common exons, and white boxes represent untranslated regions. The three alternatively spliced exons are labeled in red and indicated as iso1, iso2, and iso3.

(B) Schematic structure of Loner ISO1, ISO2, and ISO3 proteins. All three isoforms share an IQ motif, a Sec7 domain, a PH domain, and a C-terminal coiled-coil domain. Loner ISO1 is predicted to encode a 1325 amino acid protein that also contains an N-terminal coiled-coil domain. Loner ISO2 and Loner ISO3 are predicted to encode 1315 and 1210 amino acid proteins, respectively.

(C) Comparison of the molecular structures of Loner ISO1 and human Loner (hLoner), indicating the percentage of amino acid identity between each of the conserved domains.

revealed apparently normal differentiation of epidermis in *loner* mutant embryos (data not shown). Thus, the unfused myoblast phenotype in *loner* mutant embryos is likely due to a specific defect in myoblast fusion.

Since myoblast fusion is a multi-step process requiring cell-cell recognition, adhesion, alignment, and coalescence of membranes, we sought to determine which of these steps was blocked in *loner* mutant embryos. Previous studies showed that in *duf rst* double-mutant embryos, fusion-competent myoblasts extend filopodia at random orientations and are not attracted by founder cells (Ruiz-Gomez et al., 2000). In *ants/rols7* mutant embryos, however, fusion-competent myoblasts form clusters around the founder cells and extend filopodia toward their fusion targets, indicating that the fusion process arrests after the initial attraction (Chen and Olson, 2001; Menon and Chia, 2001; Rau et al., 2001). This is consistent with ANTS functioning downstream of DUF/RST in a fusion signaling pathway. Detailed analysis of *loner* mutant embryos revealed that fusion-competent myoblasts extended filopodia toward their fusion targets, as in the *ants* mutant (Figures 1C), suggesting that *loner* also functions after the initial recognition and adhesion step required for fusion. Occasionally, we observed miniature fibers that contained two nuclei, suggesting that limited fusion can occur in the absence of *loner*.

Molecular Cloning of *loner*

loner was mapped to a small chromosomal region of 78A4-B1 by deficiency mapping (see Experimental Procedures). The proximal breakpoint of the complementing deficiency *Df(3L)Pc-cp2* (78B1-2; 78D) breaks within the *knockout* gene, not only excluding it as a candidate for *loner*, but also providing us with a precise distal "molecular" boundary of the *loner* locus relative to other predicted genes. We sequenced the coding regions of several predicted genes in this chromosomal interval for potential molecular lesions in the *loner* mutant and detected point mutations in a predicted gene, CG32434. 5′ RACE experiments led to the identification of three alternatively spliced forms of CG32434, represented by three EST clones: RE02556, LP01489, and GH10594, respectively. We refer to these forms of *loner,* which vary only in their first exons, as isoforms 1, 2, and 3 (Figure 2A). Both *loner* alleles harbored nonsense mutations in the third exon, a region common to all three isoforms. $loner^{T1032}$ contained a C to T mutation that changed glutamine(Q)-414 of the predicted ISO1 (Q402 of ISO2 or Q299 of ISO3) to a stop codon. Remarkably, $loner^{T1057}$ contained a C to T mutation that changed the adjacent Q415 of ISO1 (Q403 of ISO2 or Q300 of ISO3) to a stop codon (Figures 2A and 2B).

To confirm that CG32434 corresponded to the *loner* gene, we generated transgenic flies in which each of the isoforms were expressed under the control of the UAS promoter. Expression of any of the three isoforms in the presence of the mesodermal specific *twi-GAL4* driver completely rescued the fusion defects in *loner* mutant embryos (Figures 3B, b–d). For embryos harboring the *iso3* transgene, no mononucleated myoblasts were observed, although there were some fiber-patterning defects (Figure 3B, d). A transgene encoding ISO2 under control of the ubiquitous tubulin promoter not only rescued the fusion phenotype of the somatic muscles, but also rescued *loner* mutants to adulthood (data not shown). These results provide conclusive evidence that CG32434 corresponds to *loner*.

Domain Structures of the Loner Protein

The three spliced forms of *loner* encode predicted proteins of 1325, 1315, and 1210 amino acids, respectively.

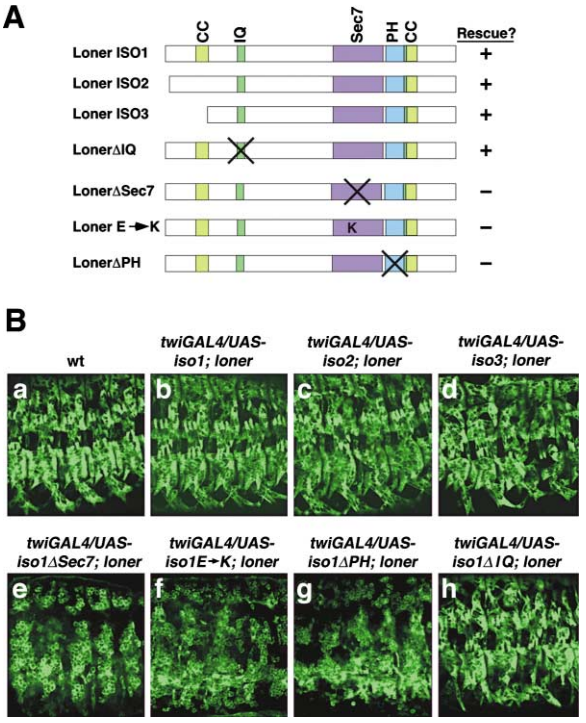

Figure 3. Phenotypic Rescue by Loner and the Requirement for its GEF Activity and PH Domain In Vivo

(A) Wild-type and mutant Loner proteins were expressed under the control of the *twi* promoter in transgenic rescue experiments. The column on the right indicates whether or not these proteins rescued the myoblast fusion defect in the *loner* mutant.

(B) Somatic musculature of *loner* mutant embryos expressing various transgenes as indicated, stained with anti-MHC. Stage 14 embryos are shown. Note that all three isoforms, *iso1* (b), *iso2* (c), *iso3* (d), as well as *iso1ΔIQ* (h), rescued the myoblast fusion phenotype of the *loner* mutant embryo. However, *iso1ΔSec7* (e), *iso1Sec7E→K* (f), and *iso1ΔPH* (g) did not rescue the mutant phenotype.

Their domain homologies suggest that Loner is a GEF (Figure 2B). All three protein isoforms contain a Sec7 domain, an adjacent pleckstrin homology (PH) domain, a C-terminal coiled-coil domain, and an IQ-motif. ISO1 also contains a coiled-coil domain at its N terminus. Sec7 domains are regions of ~200 amino acids with strong homology to the yeast protein Sec7p (Shevell et al., 1994; Morinaga et al., 1997). Sec7 domains possess GEF activity toward a family of ubiquitously expressed small GTPases called ADP-ribosylation factors (ARFs), which have been implicated in a variety of vesicular transport and cytoskeleton rearrangement processes in eukaryotic cells (Moss and Vaughan, 1998; Chavrier and Goud, 1999; Donaldson and Jackson, 2000; Jackson and Casanova, 2000). PH domains can bind to negatively charged phospholipids of cell membranes and are able to enhance GEF activity (Chardin et al., 1996; Paris et al., 1997). IQ motifs are believed to mediate binding to calmodulin (Rhoads and Friedberg, 1997).

Previously identified members of the Sec7 family are subdivided into two major classes based on sequence similarities and functional differences: high molecular weight GEFs (>100 kDa) and low molecular weight GEFs (45–50 kDa) (Jackson and Casanova, 2000). Members of the former class have orthologs in all eukaryotes and are probably involved in evolutionarily conserved aspects of membrane dynamics and protein transport. Members of the latter class do not have orthologs in *S. cerevisiae*, suggesting a function specific to higher eukaryotes. Loner shares a common domain structure with the low molecular weight Sec7 GEFs, containing a PH domain besides the Sec7 domain. However, unlike the other members of this class, Loner has a much higher predicted molecular weight of over 100 kDa.

Database searches identified a highly homologous putative mouse Loner protein of 916 amino acids (mCP20090) and a potential human Loner protein of 963 amino acids (hCP438181) sharing 65% identity in the Sec7 domain, 59% identity in the PH domain, and 65% identity in the IQ domain with the fly protein (Figure 2C). The mouse and human Loner proteins share 94% amino acid identity. A human EST, KIAA0763, previously named ARF-GEP$_{100}$ (Someya et al., 2001), is a shorter form of the human Loner lacking 122 amino acids at the N terminus. There is also a closely related predicted *C. elegans* protein, 4E572, with an IQ motif and Sec7 domain, but without a PH domain. Loner and its orthologs represent a unique high molecular weight subclass of Sec7 GEFs that contain both Sec7 and PH domains.

Expression Pattern of *loner*

The embryonic expression pattern of *loner* was examined by in situ hybridization. *loner* exhibits a dynamic expression pattern during the early stages of embryogenesis (Figures 4A–4F). It is expressed in a "gap"-like pattern at stage 4 (Figure 4A). Mesodermal expression of *loner* is initiated at embryonic stage 11, at the onset of fusion (Figure 4B). It is also expressed in the neuroectoderm. As germ band shortening proceeds, the somatic mesodermal expression persists until stage 14 (Figures 4C–4E). When fusion is completed at stage 15, *loner* is no longer expressed in the mesoderm. Instead, strong expression of *loner* persists in the embryonic CNS (Figures 4F). The temporal expression of *loner* in the somatic mesoderm coincides precisely with the fusion process, consistent with its requirement for myoblast fusion.

Loner Is Localized to Discrete Cytoplasmic Foci in Founder Cells

The subcellular distribution of the Loner protein in muscle cells was determined by double-labeling experiments with anti-Loner and anti-β-galactosidase (β-gal) antibodies using the *rP298* enhancer trap line, which carries a P[LacZ] element insertion in the 5′ promoter of the founder cell-specific *duf* gene (Ruiz-Gomez et al., 2000). Confocal microscopy demonstrated that Loner was localized to the *lacZ*-expressing founder cells (Figure 4G). Furthermore, Loner protein expression was increased in *Notch* (*N*) mutant embryos (Figure 4K), which produce excess founder cells (Corbin et al., 1991; Fuerstenberg and Giniger, 1998; Rusconi and Corbin, 1998). Interestingly, Loner is a cytoplasmic protein that aggregates to discrete foci (Figures 4G and 4J). The punctate appearance of Loner staining is reminiscent of that of ANTS/ROLS7, a founder cell-specific adaptor molecule

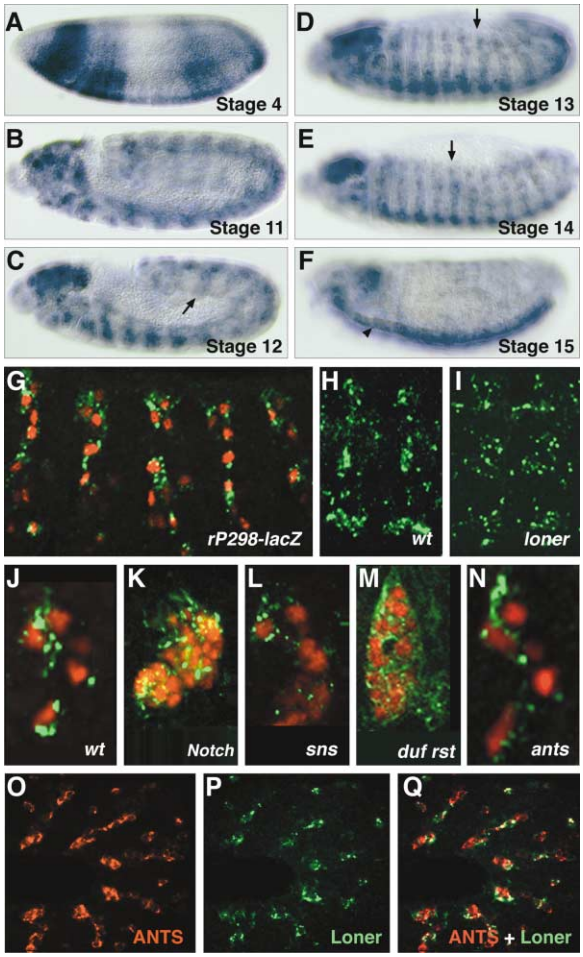

Figure 4. *loner* Expression during Embryogenesis and Subcellular Localization of the Loner Protein in Muscle Founder Cells

(A-F) Expression of *loner* transcripts in wild-type embryos detected by RNA in situ hybridization.

(A) At stage 4, *loner* is expressed in several stripes along the anteroposterior axis of the embryo.

(B and C) At stage 11 and 12, *loner* expression is seen in the neuroectoderm and begins to be expressed in the somatic mesoderm (arrow).

(D and E) At stage 13 and 14, *loner* expression is clearly seen in the somatic mesoderm (arrow). It is also expressed in the embryonic CNS, which is not in focus.

(F) At stage 15, *loner* expression disappears from the somatic mesoderm. However, its expression in the CNS persists (arrowhead).

(H and I) Localization of ANTS in stage 13 wild-type (H) and *loner* mutant (I) embryos. Muscle cells in two hemisegments are shown. Note that the punctate subcellular localization of ANTS, labeled in green, remained the same in the *loner* mutant embryo as in wild-type.

(G and J–N) Confocal images of stage 13 wild-type and mutant embryos showing the subcellular localization of Loner protein.

(G) An embryo carrying *rP298-lacZ* and double-labeled with anti-Loner (green) and anti-β-gal (red) antibodies. Muscle cells in five hemisegments are shown. Loner staining appears as discrete foci associated with founder cell nuclei.

(J–N) The nuclei of a subset of founder cells were labeled by anti-Nautilus (NAU) (red). A cluster of muscle cells within a single hemisegment is shown in each image.

(J) Loner expression is associated with founder cells in a wild-type embryo.

(K) In a *Notch* mutant embryo, where there are an increased number of founder cells, elevated Loner expression is seen associated with the founder cells.

(L and N) In *sns* and *ants* mutant embryos, the punctate pattern of Loner expression remains unchanged.

(M) In a *duf rst* mutant embryo, Loner staining is distributed throughout the cytoplasm and appears to outline the founder cell nuclei. Note that there is still some punctate staining of Loner.

(O–Q) Confocal images of a stage 11 embryo showing the expression of ANTS (red in O and Q) and Loner (green in P and Q). Muscle cells in six hemisegments during the germ band extension stage are shown. Note that Loner colocalized with ANTS at some foci (yellow), but not the others.

that is localized to sites of muscle cell fusion (Chen and Olson, 2001; Menon and Chia, 2001).

Loner Is Recruited to the Cell Membrane by Founder Cell-Specific Receptors

The punctate distribution of Loner prompted us to ask if its subcellular localization was, like ANTS, dependent on fusion receptors (Chen and Olson, 2001; Menon and Chia, 2001). To address this question, we established a cell-culture-based assay in S2 cells to investigate the interactions between fusion receptors and downstream components of the fusion-signaling cascade. When expressed alone, DUF, a homophilic adhesion molecule (Dworak et al., 2001), localized to membrane regions between adhering cells, whereas ANTS localized in the cytoplasm of S2 cells (Figures 5A and 5G). However, when DUF and ANTS were coexpressed in S2 cells, ANTS colocalized with DUF at the membrane region between adhering cells (Figures 5H and 5I). The recruitment of ANTS by DUF to cell-cell contacts in this assay agrees with previous studies showing that the subcellular localization of ANTS is dependent on DUF function (Chen and Olson, 2001; Menon and Chia, 2001). Using this cell-culture-based assay, we examined the potential interaction between DUF and Loner. While Loner localized to distinct foci in the cytoplasm of S2 cells when expressed alone (Figure 5J), it was recruited to membrane regions of cell-cell contacts in the presence of DUF (Figures 5B and 5C). This recruitment is highly specific, since coexpressing Loner with another cell adhesion molecule, Delta, did not result in membrane localization of Loner (Figure 5K). Thus, Loner, like ANTS, can be recruited to membrane regions of cell-cell contact by DUF. RST, a DUF-related fusion receptor that plays redundant roles with DUF in myoblast fusion (Strunkelnberg et al., 2001), was also able to recruit Loner and ANTS to cell-cell contacts in the S2 cell assay (Figures 5D–5F, and data not shown). We have not detected direct interactions between Loner and DUF/RST (data not shown), suggesting the involvement of additional intermediary proteins.

To substantiate a role for DUF/RST in the subcellular localization of Loner, we examined Loner protein in homozygous mutant embryos of a small deficiency, $Df(1)w^{67k30}$, which removed both *duf* and *rst*. As shown in Figure 4M, instead of localizing to discrete foci in founder cells, at least a portion of the Loner protein was distributed throughout the cytoplasm and appeared as rings that outlined the founder cells in *duf rst* double-mutant embryos. Interestingly, some discrete foci of Loner protein remained in founder cells, suggesting the existence of different pools of Loner protein in the cyto-

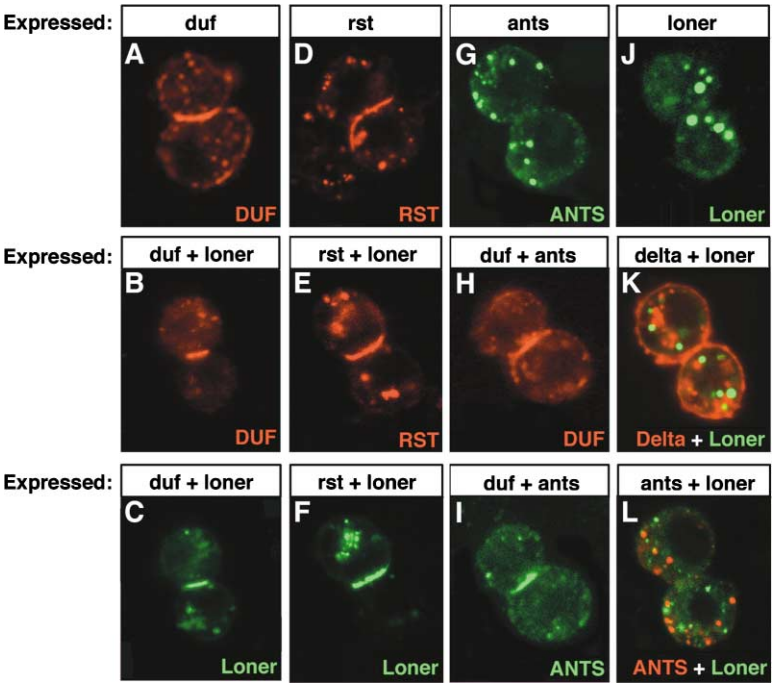

Figure 5. Loner and ANTS Are Recruited to the Cell Membrane by Founder Cell-Specific Receptors, DUF and RST, in Transfected S2 Cells

Transgenes expressed are indicated above each image.

(A, D, G, and J) Localization of DUF (A), RST (D), ANTS (G), and Loner (J) in S2 cells when they were expressed alone. Cells were stained with anti-V5 to visualize V5-tagged DUF (red) and RST (red), anti-FLAG for FLAG-tagged Loner (green), and anti-MYC for MYC-tagged ANTS (green). Note that DUF and RST localized at the membrane region between two adhering cells, whereas ANTS and Loner localized in the cytoplasm of two randomly adjacent cells.

(B and C) Localization of DUF (B) and Loner (C) when they were coexpressed.

(E and F) Localization of RST (E) and Loner (F) when they were coexpressed. Note that in both cases, Loner was recruited to the membrane region between two adhering cells.

(H and I) Localization of DUF (H) and ANTS (I) when they were coexpressed. Note that ANTS, like Loner, was recruited to the cell membrane between two adhering cells.

(K) Localization of Delta (red) and Loner (green) when they were coexpressed in S2 cells. Note that Loner still remained cytoplasmic, even though Delta localized at the membrane of the two adhering cells.

(L) Loner (green) and ANTS (red) do not colocalize when coexpressed in S2 cells.

plasm. On the other hand, Loner localization was not affected in embryos lacking the transmembrane protein SNS, expressed specifically in fusion-competent myoblasts (Figure 4L). These results are consistent with the recruitment of Loner by DUF and RST to discrete loci at the founder cell membrane in vivo.

The Subcellular Localization of Loner Is Independent of ANTS

It is intriguing that Loner and ANTS share several common features with respect to their intracellular localization: both are expressed in discrete foci in founder cells; both can be recruited to the cell membrane by DUF and RST in S2 cells; and the subcellular localization of both is altered in *duf rst* mutant embryos. However, they appear to be in different foci when coexpressed in S2 cells (Figure 5L). This prompted us to investigate if Loner and ANTS are localized to the same or distinct foci in founder cells in vivo. Antibody double-labeling experiments revealed that Loner and ANTS partially colocalized in founder cells, with some foci containing both proteins and others containing either one or the other of the two proteins (Figures 4O–4Q). Given that the ANTS-positive foci represent fusion sites (Menon and Chia, 2001), this result suggests that at least some of the Loner-positive foci also correspond to sites of muscle cell fusion.

To investigate if the localization of Loner and ANTS is interdependent, we examined the subcellular localization of Loner in *ants* mutant embryos and the localization of ANTS in *loner* mutant embryos. As shown in Figures 4I and 4N, Loner and ANTS maintain their pattern of subcellular localization in the absence of the other protein. These results, combined with observations that the subcellular localization of ANTS and Loner are both dependent on the fusion receptors DUF/RST, suggest that DUF/RST recruit ANTS and Loner independently to the subcellular sites of fusion and that Loner and ANTS might function in parallel downstream of DUF and RST during myoblast fusion (see Figure 7).

The GEF Activity of Loner Is Required for Its Function In Vivo

To determine the functional significance of the conserved domains of Loner, we examined the in vivo activities of a series of mutant Loner proteins. Wild-type Loner, but not a Sec7-deletion mutant, or a "GEF-dead" Loner mutant containing an E-to-K point mutation in the conserved GEF domain (Shevell et al., 1994), rescued the *loner* mutant phenotype (Figure 3B, b, e, and f). Using the same rescue assays, we found that deletion of the PH domain also abolished the ability of Loner to rescue the myoblast fusion phenotype (Figure 3B, g), whereas deletion of the IQ motif did not affect Loner activity (Figure 3B, h). Taken together, these results show that the Sec7 and PH domains, but not the IQ domain, are essential for Loner function in the context of myoblast fusion.

Loner Has Specific Activity toward the Small GTPase ARF6 In Vitro

The Sec7 family of GEFs regulates the ARF family of small GTPases. Mammalian ARFs can be divided into class I (ARF1-3), class II (ARF4, 5), and class III (ARF6). Among these, ARF6 has been implicated in endocytosis, membrane recycling, and cytoskeletal rearrangement (Chavrier and Goud, 1999; Donaldson and Jackson, 2000). Several low molecular weight Sec7 GEFs related to Loner, such as cytohesins, EFA6, as well as ARF-

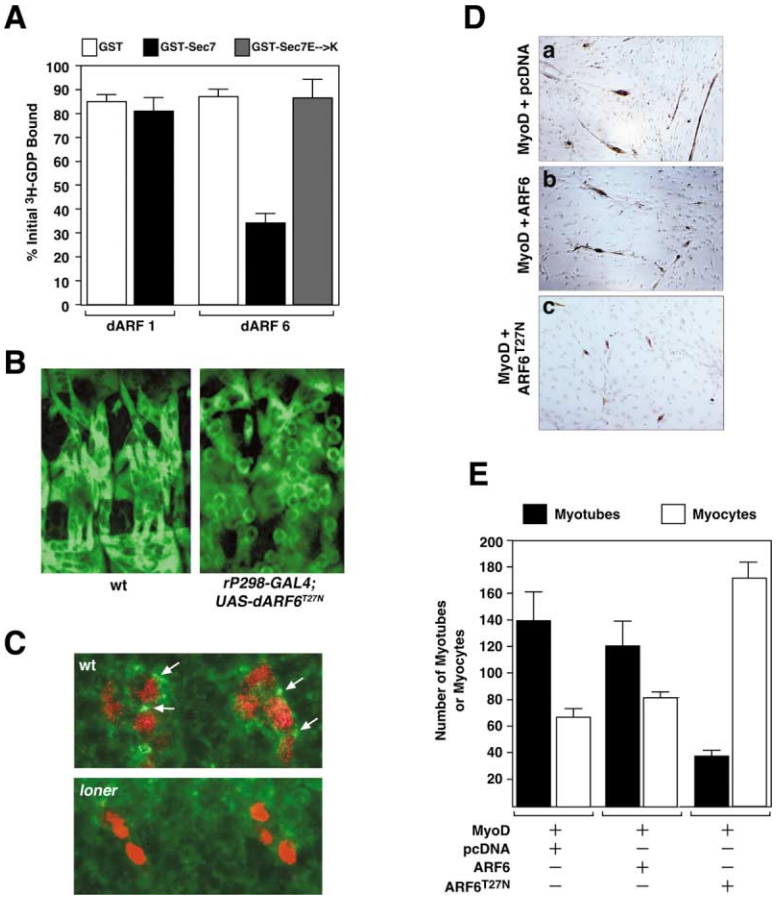

Figure 6. GTPase Target of Loner and Its Involvement in *Drosophila* and Mammalian Myoblast Fusion

(A) Loner GEF stimulates guanine nucleotide release on dARF6, but not dARF1. Activity of 2 ug of GST alone, GST-Sec7, and GST-Sec7E→K on ^{3}H-GDP release from GST fusion proteins of *Drosophila* ARF family GTPases, dARF6 and dARF1, respectively. The activity is expressed as the percent of initial ^{3}H-GDP remaining bound after 20 min incubation.

(B) Somatic musculature of a stage 14 wild-type embryo and a same stage embryo carrying the *rP298-GAL4* driver and *UAS-dARF6^{T27N}* transgene, visualized by a *MHC-tauGFP* reporter. Note that there are many unfused myoblasts in the latter embryo.

(C) Localization of Rac protein (green) in stage 13 wild-type and *loner* mutant embryos. The nuclei of a subset of founder cells were labeled by anti-NAU staining (red). High levels of Rac protein aggregates are observed in discrete foci, a few of which are indicated by arrows, along the founder cell membrane in wild-type, but not *loner* mutant, embryos.

(D) Regulation of myogenesis by ARF6. 10T1/2 fibroblasts were cotransfected with expression vectors for MyoD and pcDNA as a control (a), ARF6 (b), and ARF6^{T27N} (c). Fibroblasts converted to differentiated myotubes or myocytes were marked by anti-myosin antibody.

(E) The number of myosin-positive myotubes (black bar) and mononucleated myocytes (white bar) in cultures cotransfected of MyoD with pcDNA, ARF6, and ARF6^{T27N}, respectively. The myotubes counted here contained at least three fused muscle cells. Values represent the mean ± SD from at least three experiments.

GEP_{100}, have been shown to activate ARF6 in vitro (Frank et al., 1998; Franco et al., 1999; Langille et al., 1999; Someya et al., 2001), suggesting that ARF6 may be a downstream small GTPase for Loner. Searches of *Drosophila* genome sequence (Adams et al., 2000) identified an apparent ARF6 homolog, *Arf51F*, which shares 97% amino acid identity with mammalian ARF6. For simplicity, we will refer to Arf51F as dARF6. To test if Loner functions as a GEF for dARF6, we carried out in vitro GDP release assays using GST fusion proteins containing dARF6 and GST fusion proteins containing the wild-type Sec7 domain of Loner or a Sec7 domain with an E-to-K mutation that is known to abolish GEF activity (Shevell et al., 1994). As shown in Figure 6A, the wild-type Sec7 domain, but not the GEF-dead Sec7 domain, catalyzed GDP/GTP exchange of dARF6. This activity was highly specific, since no significant GDP release was detected using the highly related GTPase dARF1 (*Arf79F*) as the substrate (Figure 6A). These results demonstrate that Loner functions as a specific dARF6 GEF in vitro.

dARF6 Is Involved in Myoblast Fusion

The biochemical link between Loner and dARF6, combined with the essential requirement for the GEF activity of Loner in myoblast fusion, prompted us to investigate whether dARF6 might also be involved in the same process. In situ hybridization showed that dARF6 is ubiquitously expressed in the embryo (data not shown). Since no loss-of-function mutant of dARF6 exists, we engineered transgenic flies carrying a dominant-negative form of dARF6 (dARF6^{T27N}) (D'Souza-Schorey et al., 1998) under control of the UAS promoter. Expression of the mutant ARF6 in founder cells severely perturbed myoblast fusion throughout the internal layer of somatic mesoderm, although external muscle fibers were formed at their characteristic positions (Figure 6B). This phenotype is similar to, but less severe than that of *loner* mutant embryos. The weaker phenotype resulting from dARF6^{T27N} expression could be due to incomplete inhibition of endogenous dARF6 by dARF6^{T27N} or, alternatively, the relatively late expression of the *rP298-GAL4* line, which does not start until stage 12, when myoblast fusion has already initiated for many muscle fibers. Taken together, our results suggest that ARF6 is an essential downstream mediator of Loner activity during myoblast fusion in the *Drosophila* embryo.

The Loner/ARF6 Module Is Required for the Subcellular Localization of Rac

To further understand the function of Loner/ARF6 in myoblast fusion, we investigated whether the Loner/

ARF6 module impinged upon the ANTS→MBC→Rac pathway. Previous studies in mammalian cultured cells have suggested that ARF6 is involved in localizing Rac to the plasma membrane, a prerequisite for Rac's function in cytoskeletal rearrangement (Radhakrishna et al., 1999). Since Rac is also required for myoblast fusion in *Drosophila*, we tested if the Loner/ARF6 module is required for the proper localization of Rac in founder cells. In wild-type embryos, high levels of Rac protein are observed in discrete foci along the founder cell membrane, which correspond to sites of fusion (Figure 6C and data not shown). However, in *loner* mutant embryos, the specific aggregation of Rac at the fusion sites was no longer observed (Figure 6C). These data suggest that the Loner/ARF6 module converges with the ANTS→MBC→Rac pathway at the small GTPase level, and Loner/ARF6 are required, independent of ANTS, for the proper subcellular localization of Rac in founder cells.

Dominant-Negative ARF6 Disrupts Mammalian Myoblast Fusion

To determine if a similar ARF6-mediated pathway might control fusion of mammalian muscle cells, we investigated whether expression of a dominant-negative form of ARF6, ARF6^{T27N}, could interfere with muscle differentiation in vitro, which is dependent on MyoD (Davis et al., 1987). When 10T1/2 fibroblasts were transfected with MyoD and switched to differentiation medium, the transfected cells acquired a myoblast fate and differentiated into myotubes (Figures 6D, a and 6E). Coexpression of MyoD with wild-type ARF6 did not significantly affect the differentiation of 10T1/2 cells into myotubes (Figure 6D, b and 6E). However, coexpression of ARF6^{T27N} with MyoD severely decreased the efficiency of myotube formation, whereas myocyte differentiation was not affected (Figures 6D, c and 6E). Only occasionally could myotubes be observed in cells coexpressing MyoD and ARF6^{T27N}. This phenotype, in which myosin heavy chain expression is induced but fusion is specifically blocked, is analogous to the phenotype of *loner* mutant embryos or embryos expressing *dARF6*^{T27N}. Thus, ARF6 appears play an important role in mammalian myoblast fusion, as in *Drosophila*.

Discussion

Through a genetic screen for regulators of muscle development in *Drosophila*, we discovered Loner, a GEF required for myoblast fusion. Our results ascribe four interdependent functions to Loner: (1) it acts as a downstream effector of myoblast fusion receptors; (2) it recruits ARF6 to subcellular sites of fusion; (3) it promotes guanine nucleotide exchange by ARF6; and (4) it impinges on the ANTS→MBC→Rac pathway at the small GTPase level. These findings establish Loner and ARF6 as key components of a cellular apparatus governing myoblast fusion and suggest the involvement of ARF-GEF signaling in intercellular fusion of other cell types.

Loner, a Member of the ARF-GEF Family

The ARF-GEFs constitute a large and diverse family of proteins (Moss and Vaughan, 1998; Chavrier and Goud, 1999; Donaldson and Jackson, 2000; Jackson and Casanova, 2000). Despite extensive molecular and biochemical characterization of ARF-GEFs in yeast and mammalian cell culture, their functions in multicellular organisms are largely unknown. The only exception, to our knowledge, is GNOM/Emb30, a high molecular weight GEF specific for ARF1 that is required for the establishment and maintenance of cell polarity in *Arabidopsis* (Shevell et al., 1994; Geldner et al., 2003). At present, none of the low molecular weight ARF-GEFs has been implicated in a physiological process in vivo.

The Loner protein contains a Sec7 domain, a PH domain and a coiled-coil domain. Such a domain organization is reminiscent of low molecular weight ARF-GEFs. However, Loner is distinguished from conventional low molecular weight ARF-GEFs by its high molecular weight (>100 kDa), as well as additional features such as the presence of an IQ motif at the N terminus. Therefore, Loner and its mammalian homologs define a distinct subclass of ARF-GEFs. Our studies have revealed a physiological function for this subclass of ARF-GEFs and have provided insights into their structure-function relationships.

The Sec7 domain of Loner has specific GEF activity toward *Drosophila* dARF6, a finding consistent with biochemical studies of ARF-GEP$_{100}$, a human homolog of Loner (Someya et al., 2001). Importantly, the GEF activity of Loner is required for myoblast fusion, since deletion of the Sec7 domain or a "GEF-dead" version of the Sec7 domain completely abolished the ability of Loner to rescue the fusion defects in *loner* mutant embryos. The PH domain is also required for Loner function, as revealed by the loss of fusigenic activity of a Loner deletion mutant lacking this region. The PH domain of the low molecular weight ARF-GEFs such as cytohesins has been implicated in phosphoinositide binding and targeting to the plasma membrane (Chardin et al., 1996; Paris et al., 1997).

The Role of Loner and ARF6 in Myoblast Fusion

Myoblast fusion requires the initial recognition and adhesion between founder cells and fusion-competent myoblasts, followed by cytoskeleton rearrangements that lead to the proper alignment of the two populations of cells and eventual membrane coalescence. A pathway involving ANTS and MBC has been proposed to transduce fusion signals from the founder cell-specific surface receptors DUF/RST to the small GTPase, Rac, which controls actin cytoskeleton rearrangement (Hall, 1998; Chen and Olson, 2001). Our current studies have revealed additional components of the cellular apparatus downstream of the fusion receptors and suggested the existence of a multiprotein "fusion complex" at myoblast fusion sites. The small GTPase, ARF6, and its GEF, Loner, act as essential effectors in this cellular apparatus (Figure 7).

Several lines of evidence suggest that Loner acts downstream of the founder cell-specific receptors, DUF and RST. First, Loner is recruited to the cell membrane by DUF/RST in cell-culture assays, mimicking the behavior of ANTS, which acts downstream of DUF/RST (Chen and Olson, 2001). Second, *loner* encodes a cytoplasmic protein that is expressed at sites of fusion in founder cells. Third, the subcellular localization of a sub-

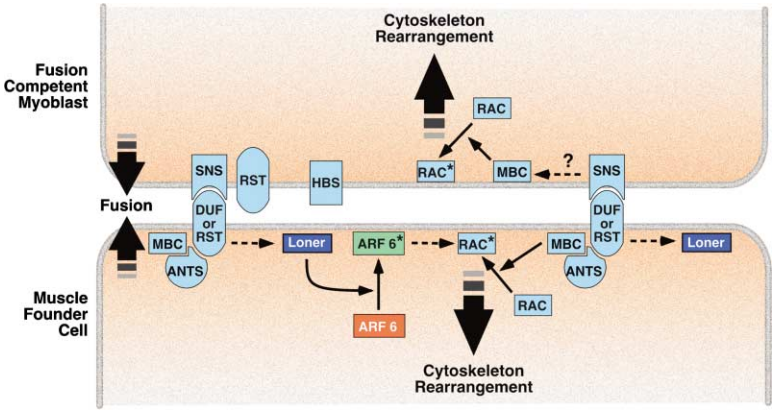

Figure 7. Model for Loner and ARF6 in Myoblast Fusion

We propose that Loner is recruited to the cell membrane by founder cell-specific receptors, DUF/RST, likely through interaction with adaptor protein(s). DUF/RST organize a multiprotein "fusion complex," including ANTS and Loner, at the sites of fusion. The membrane recruitment of Loner is independent of the adaptor protein ANTS. Aggregation of Loner at the sites of fusion in turn recruits and activates ARF6 via its Sec7 domain. The Loner/ARF6 module impinges on the ANTS→MBC→Rac pathway at the small GTPase level by controlling the proper membrane localization of Rac, a prerequisite for actin cytoskeleton rearrangement required for cell fusion. Activated forms of ARF6 and Rac are marked as ARF6* and Rac*, respectively.

set of Loner is altered in embryos lacking DUF/RST. Lastly, in *duf rst* mutant embryos, myoblast fusion is blocked at the initial recognition and adhesion between fusion-competent myoblasts and their targets (Ruiz-Gomez et al., 2000), while myoblast fusion is blocked at a later step in *loner* mutant embryos (this study). Such a temporal order is consistent with Loner functioning downstream of DUF/RST.

We showed previously that the founder cell-specific adaptor protein ANTS localized to fusion sites through direct physical interaction with the fusion receptors, DUF and RST (Chen and Olson, 2001; data not shown). We demonstrate here that Loner is recruited by DUF/RST to the cell membrane independent of ANTS. Thus, the formation of the "fusion complex" is initially organized by the founder cell receptors. Our studies also suggest that Loner functions in parallel to ANTS, since Loner and ANTS are recruited independently to the cell membrane by DUF/RST and the localization of ANTS and Loner is independent of each other.

We propose that a major function of Loner is to recruit its downstream GTPase, ARF6, to fusion sites defined by DUF/RST. Given the role of ARF6 in cytoskeleton organization (Chavrier and Goud, 1999), activation of ARF6 by Loner at subcellular sites of fusion is likely to be essential for cytoskeleton rearrangement, a prerequisite for proper cell alignment in the fusion process.

Small GTPases in Myoblast Fusion

It is intriguing that two small GTPases, ARF6 and Rac, are both required for myoblast fusion. Our data suggest that the Loner/ARF6 module impinges on the ANTS→MBC→Rac pathway at the small GTPase level, and Loner/ARF6 are required for the proper localization of Rac to the founder cell membrane, which in turn induces actin cytoskeleton rearrangements required for fusion. These findings are consistent with studies in mammalian cells demonstrating strong interactions between ARF6 and Rac in regulating the cortical actin cytoskeleton. For example, ARF6 and Rac can bind to the same protein, POR-1, and ARF6 is required for the membrane localization of Rac (D'Souza-Schorey et al., 1997; Radhakrishna et al., 1999; Boshans et al., 2000). However, our findings do not exclude other possible roles of ARF6, for example, in its demonstrated activity toward enzymes responsible for lipid modification, such as PIP5-kinase and PLD (Brown et al., 2001), or in regulated secretion events that it has been associated with in mammalian systems (D'Souza-Schorey et al., 1998).

Implications Beyond *Drosophila* Myogenesis

Given the evolutionary conservation of muscle developmental control mechanisms (Wakelam, 1985; Knudsen, 1992; Doberstein et al., 1997), it is likely that homologs of genes involved in *Drosophila* myoblast fusion play similar roles in mammalian skeletal muscle development. In this regard, we showed previously that a mouse homolog of *ants* is expressed in the embryonic mesoderm at the time of myoblast fusion, suggesting its potential involvement in myogenesis (Chen and Olson, 2001). Here, we show that ARF6 is involved in mammalian myoblast fusion, suggesting that the Loner/ARF6 module may play conserved roles in evolution.

Cell fusion is a universal and evolutionarily ancient process required for development of multicellular organisms. Despite the diversity of cell types that undergo cell-cell fusion (e.g., sperm-egg, osteoclasts, hematopoietic stem cells, muscle cells), the cellular events involved in this process—cell recognition, adhesion, and membrane merger—are common to all these cell types, suggesting shared cellular mechanisms. In *Drosophila* muscle cells, Loner and ARF6 are controlled by the cell surface fusion receptors, DUF and RST. However, Loner/ARF6 are expressed in a wide range of cell types. Thus, they may represent a general fusigenic mechanism coopted by different upstream effectors to control intercellular fusion of diverse cell types. The recognition that intercellular fusion is controlled by a G protein-dependent mechanism involving Loner and ARF6 provides interesting opportunities for modulating this process in a variety of therapeutic settings.

Experimental Procedures

Genetics

Mutations disrupting myoblast fusion were isolated in a genetic screen for muscle development (E.H.C. and E.N.O., unpublished data). *loner* mutants were initially defined by a lethal complementation group of two EMS-induced alleles, $loner^{T1032}$ and $loner^{T1057}$. The third chromosome deficiency kit, DK3, was used in complementation tests to map these mutants to the 77F3 to 78C8-9 region, de-

fined by *Df(3L)ME107*. Subsequently, the *loner* mutants were mapped by overlapping small deficiencies in this region, kindly provided by Minx Fuller. *Df(3L)Pc-MK* (78A2;78C9) did not complement either *loner* allele, whereas *Df(3L)ri-XT1* (77E2; 78A4) and *Df(3L)Pc-cp2* (78B1-2; 78D) complemented both *loner* alleles, delimiting the *loner* locus to 78A4-B1. Both alleles result in identical phenotypes as homozygous embryos or transheterozygous embryos over deficiency *Df(3L)ME107*. Therefore, we infer that they both behave as null alleles.

The *rP298-lacZ* stock was generously provided by Akinao Nose, the *rP298-GAL4* driver by Devi Menon, the $sns^{40\text{-}49}$/*CyO* by Renate Renkawitz-Pohl. $Df(1)w^{67k30}$, a small deficiency deleting both *duf* and *rst*, N^{81k}, and *twi-GAL4* were obtained from the Bloomington stock center.

In overexpression studies using the *rP298-GAL4* driver, males carrying *rP298-GAL4* were crossed with females carrying various pUAST constructs.

Molecular Biology

Full-length EST clones RE02556, LP01489, and GH10594 (Berkeley *Drosophila* Genome Project [BDGP]) were obtained from Research Genetics.

DNA sequences of *loner* alleles were determined by directly sequencing PCR products amplified from genomic DNA obtained from homozygous mutant embryos selected by their lack of armadillo-GFP expression, which was carried on the balancer chromosome. When a mutation was uncovered, PCR and sequencing were repeated to confirm that the mutation was not due to PCR errors.

loner transgenes were prepared using standard subcloning procedures. For rescue constructs with full-length *loner* cDNAs, EcoRI fragments including *loner iso1*, *iso2*, or *iso3* from their respective EST clones were subcloned into transformation vectors, pUAST and S102 (containing a tubulin promoter), respectively. *loner* deletion and point-mutation constructs were prepared using standard PCR procedures to introduce the necessary changes on their original EST clones (Stratagene) and subcloned into pUAST and S102, respectively.

dARF6 (*Arf51F*) transgenes were prepared by amplifying the dARF6 coding sequence from total embryonic cDNA. Standard PCR procedures were used to introduce a T27→N point mutation (Stratagene). Wild-type and mutant dARF6 were then subcloned into the pUAST vector.

For bacterial expression, the coding sequences for Loner Sec7, Loner Sec7E→K, dARF6, and dARF1 were amplified from the *loner iso1*, *loner iso1E→K*, or total embryonic cDNA, and cloned in frame into pGEX-2T (Pharmacia).

Constructs used for S2 cell transfection were prepared as follows. FLAG-tagged Loner: *loner iso1*, *iso2*, and *iso3* were amplified by PCR with the 5′ primer containing sequences encoding a FLAG tag. They were then subcloned into the pAc-V5 His expression vector (Invitrogen). V5-tagged RST: the full-length coding regions of *rst* were amplified by PCR from embryonic cDNA and cloned in-frame into the pAc-V5 His vector.

Constructs used for 10T1/2 cell transfection were prepared as follows. The ARF6 and $ARF6^{T27N}$ cDNAs, generously provided by Michael Roth, were amplified by PCR with the 3′ primer containing sequences encoding a MYC tag. They were then subcloned into the pcDNA expression vector (Invitrogen).

All constructs were verified by sequence analysis.

Biochemistry

GST fusion proteins were prepared according to standard protocols (Pharmacia). Guanine nucleotide exchange assays were performed using bacterially expressed GST fusion proteins as described in Debant et al. (1996). Each assay was performed in triplicate.

Cell Culture and Transfection

Drosophila S2 cells were transfected using the Effectene transfection reagent (Qiagen) according to manufacturer's instructions. In the case of Hm-Delta, expression of Delta was induced with 0.7 mM $CuSO_4$ a day after the initial transfection. Cells were subjected to immunocytochemistry two days after transfection.

Mouse 10T1/2 fibroblasts were maintained at low density in DMEM with 10% fetal bovine serum (FBS). Transfections were conducted with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. 0.4 ug of plasmid was used for each well in a 12-well plate. Two days after transfection, cells were shifted to a differentiation medium (DMEM with 2% horse serum). Cells were subjected to immunocytochemistry five days after differentiation.

Immunohistochemistry

In situ hybridization of *Drosophila* embryos was performed using standard protocols (Tautz and Pfeifle, 1989). DIG-labeled probe was synthesized with the common coding region of the three isoforms of *loner*.

Rat polyclonal Loner antisera were generated against a carboxy-terminal peptide RIPGRERKASRTDENGRS (Bio-Synthesis) and used at 1:300 in combination with the TSA fluorescence system (NEN Life Sciences). Embryo-staining procedures were performed as described (Patel, 1994), using the following additional antibodies: rabbit anti-MHC (1:1000) and mouse anti-MHC (1:10) (Kiehart and Feghali, 1986); rabbit anti-DMEF2 (1:800) (Nguyen et al. 1994); rabbit anti-KR (1:3000) (Gaul et al., 1987); rabbit anti-NAU (1:800) (Keller et al., 1997); mouse anti-Rac1 (1:300) (BD Transduction Laboratories); rabbit anti-β-gal (1:1500) (Cappel); and mouse anti-β-gal (1:1000) (Promega). Secondary antibodies used were: Cy3 goat anti-rabbit (1:300) (Jackson) and biotinylated antibodies made in goat (1:300) (Vector Labs).

S2 cells were fixed with 4% paraformaldehyde and stained with the following primary antibodies: mouse anti-V5 (1:1000) (Invitrogen); rabbit anti-MYC (1:300) (Santa Cruz); rabbit anti-FLAG (1:500) (Sigma); and mouse anti-Delta (1:20) (Developmental Studies Hybridoma Bank). Secondary fluorochrome-conjugated antibodies were used at 1:200 (Jackson).

10T1/2 cell myogenic conversion assays were performed as described (Lu et al., 2000). Mouse antiskeletal myosin (MY32) (1:400) (Sigma) was used to stain the differentiated skeletal muscle cells.

Fluorescent images were collected on a LSM410 Zeiss confocal microscope and were processed with Adobe Photoshop 7.

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