

# Antisocial, an Intracellular Adaptor Protein, Is Required for Myoblast Fusion in *Drosophila*

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## Summary

Somatic muscle formation in *Drosophila* requires fusion of muscle founder cells with fusion-competent myoblasts. In a genetic screen for genes that control muscle development, we identified *antisocial* (*ants*), a gene that encodes an ankyrin repeat-, TPR repeat-, and RING finger-containing protein, required for myoblast fusion. In *ants* mutant embryos, founder cells and fusion-competent myoblasts are properly specified and patterned, but they are unable to form myotubes. ANTS, which is expressed specifically in founder cells, interacts with the cytoplasmic domain of Dumbfounded, a founder cell transmembrane receptor, and with Myoblast city, a cytoskeletal protein, both of which are also required for myoblast fusion. These findings suggest that ANTS functions as an intracellular adaptor protein that relays signals from Dumbfounded to the cytoskeleton during myoblast fusion.

## Introduction

The formation of skeletal muscle requires the commitment of multipotent mesodermal stem cells to a myogenic fate, followed by the fusion of mononucleated myoblasts to form multinucleated myotubes and the patterning, morphogenesis, and innervation of mature muscle fibers (Hauschka, 1994). It has become apparent in recent years that many of the cellular and molecular events involved in skeletal muscle formation are evolutionarily conserved in vertebrates and the fruit fly *Drosophila* (Wakelam, 1985; Knudsen, 1992; Doberstein et al., 1997; Baylies et al., 1998). This conservation has made it possible to dissect the process of muscle development using *Drosophila* genetics and thereby potentially uncover regulatory genes that would otherwise be difficult or impossible to identify in vertebrate model organisms.

The somatic musculature of *Drosophila* is composed of a stereotyped, segmentally repeated pattern of 30 muscle fibers per hemisegment. Larval body wall muscle development begins during embryogenesis and can be divided into two distinct stages—myoblast fate determination and myoblast fusion (Bate, 1990, 1993). During mid-embryogenesis, a population of mesodermal cells, marked by the expression of the *twist* gene, acquires a myoblast cell fate. Subsequently, a subset of myoblasts, marked by the expression of *lethal of scute*, is selected via a lateral inhibition process to become muscle

founder cells while the remaining *twist*-expressing cells become fusion competent (for reviews, see Baylies et al., 1998; Frasch, 1999). It is believed that the founder cells serve as sources of attractant for the surrounding fusion-competent cells to fuse with them and form myotubes that typically comprise between 4 and 25 myoblasts. Thus, the founder cells act as “seeds” for the future muscle fibers to determine their position, orientation, size, and pattern of motorneuron innervation (Bate, 1990, 1993).

Electron microscopic studies have revealed that myoblast fusion is a multistep process that involves similar ultrastructural changes in vertebrate and *Drosophila* muscle cells (Wakelam, 1985; Knudsen, 1992; Doberstein et al., 1997). Based on these studies, *Drosophila* myoblast fusion can be divided into four steps, including cell-cell recognition, adhesion, alignment, and membrane fusion (Doberstein et al., 1997). First, a myoblast recognizes an appropriate cellular target for fusion, for example, a founder cell or a forming myotube. Then, the myoblast adheres to the founder cell or the myotube. At this point, a prefusion complex forms along closely apposed plasma membranes. The prefusion complex consists of groups of paired vesicles with associated electron-dense material on each side of the membrane. Later, the prefusion complex resolves into electron-dense plaques along the plasma membranes of the apposed cells. The fusing cells align along their long axes, and pores form between the apposed plasma membranes. Finally, the plasma membranes vesiculate along their shared lengths, followed by vesiculation of the apposed membranes.

Recent genetic studies have identified several genes essential for myoblast fusion in *Drosophila* (for reviews, see Paululat et al., 1999; Frasch and Leptin, 2000; Taylor, 2000; Baylies and Michelson, 2001). *dumbfounded* (*duf*) encodes a transmembrane protein with extracellular immunoglobulin (Ig) domains and is expressed in founder cells (Ruiz-Gómez et al., 2000). *sticks and stones* (*sns*), which also encodes a transmembrane protein with Ig domains, is expressed in fusion-competent cells (Bour et al., 2000). It has been suggested that DUF acts as an attractant for fusion-competent cells by interacting with the SNS protein (Frasch and Leptin, 2000). Myoblast city (MBC), a *Drosophila* homolog of human DOCK180, has been proposed to mediate changes in the cytoskeleton during myoblast fusion, since human DOCK180 has been implicated in signaling by the Rho/Rac family of GTPases to the cytoskeleton (Rushton et al., 1995; Erickson et al., 1997; Nolan et al., 1998). Another gene required for myoblast fusion is *blown fuse* (*blow*), which encodes a cytoplasmic protein with no significant sequence homology to known proteins (Doberstein et al., 1997). The structures and functions of these proteins suggest the existence of a signaling pathway for myoblast fusion in which transmembrane receptors are linked to components of the cytoskeleton. However, to date, there has been no biochemical evidence for direct

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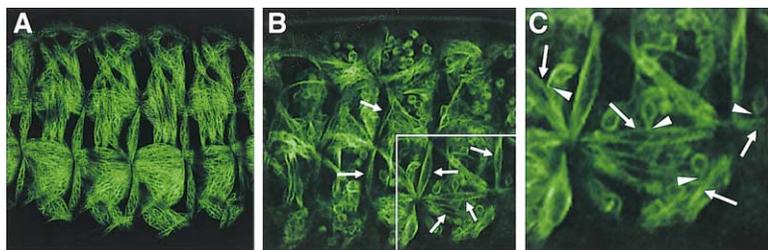


Figure 1. *ants* Function Is Required for Myoblast Fusion at a Step after Myoblast Adhesion  
A *MHC-tauGFP* reporter visualizes somatic musculature in wild-type (A) and *ants*<sup>T627</sup> embryos (B and C). Embryos are oriented with dorsal up and anterior to the left.

(A) Ventrolateral view of a portion of a wild-type embryo showing the segmentally repeated pattern of its somatic musculature. Note that *tauGFP* is localized in the cytoplasm, but not in the nuclei, of the muscle fibers. (B) Lateral view of a portion of a late stage 13 *ants*<sup>T627</sup> embryo in which myoblasts fail to fuse. Arrows point to elongated mononucleated myocytes. Note that each elongated myocyte contains a single elongated halo, which represents a single nucleus. (C) A close-up view of the boxed region in (B). Fusion-competent myoblasts extend filopodia (arrowheads) toward elongated mononucleated founder cells (arrow), suggesting that adhesion between fusion-competent myoblasts and founder cells is not affected.

fibers. Therefore, the nuclei are seen as halos within each mature fiber, and the number of halos in each fiber represents the number of myoblasts fused.

(B) Lateral view of a portion of a late stage 13 *ants*<sup>T627</sup> embryo in which myoblasts fail to fuse. Arrows point to elongated mononucleated myocytes. Note that each elongated myocyte contains a single elongated halo, which represents a single nucleus.

(C) A close-up view of the boxed region in (B). Fusion-competent myoblasts extend filopodia (arrowheads) toward elongated mononucleated founder cells (arrow), suggesting that adhesion between fusion-competent myoblasts and founder cells is not affected.

interactions between these proteins, and the mechanism whereby they cooperate to control myoblast fusion remains a mystery.

We have performed a mutagenesis screen in *Drosophila* to identify genes required for skeletal muscle development. In this paper, we present the characterization of *antisocial* (*ants*), a gene required specifically for myoblast fusion. *ants*, which is expressed in the early mesoderm and in founder cells before and during fusion, encodes a protein with multiple protein-protein interaction motifs, including ankyrin repeats, tetratricopeptide repeats (TPRs), a RING finger, and a coiled-coil domain. The ANTS protein is localized to discrete foci in the cytoplasm of founder cells. Through systematic analysis of the ability of ANTS to physically associate with all known *Drosophila* muscle fusion proteins, we found that ANTS specifically associates with DUF and MBC but not with SNS or BLOW. Furthermore, the subcellular localization of ANTS is altered in *duf* mutant embryos. These results suggest that ANTS controls myoblast fusion by serving as a linker protein between the transmembrane receptor DUF and the cytoskeleton.

## Results

### Identification of the *ants* Locus

We carried out an F2 lethal screen in *Drosophila* to identify new genes involved in skeletal muscle development (E.H.C. and E.N.O., unpublished data). To facilitate the screening process, we constructed a GFP reporter driven by the muscle-specific *myosin heavy chain* promoter (*MHC-tauGFP*), which allowed the examination of muscle morphology in live embryos (Figure 1A). We focused our initial efforts on the characterization of one complementation group on the third chromosome that contains four EMS mutant alleles, T59, T192, T321, and T627. In mutant embryos of all four alleles, the developing body wall muscles exhibit a near complete block of myoblast fusion, as revealed by the expression of the *MHC-tauGFP* reporter (Figure 1B). Based on its myoblast fusion phenotype, characterized by inappropriate interactions of myoblasts, we named this locus *antisocial* (*ants*).

In wild-type embryos, muscle MHC is expressed in muscle precursor cells and mature muscle fibers from stage 13 (Kiehart and Feghali, 1986). The fusion-competent myoblasts do not express MHC unless they have

fused with a founder cell or an existing myotube. In *ants* mutant embryos, mature, multinucleated muscle fibers are absent. Instead, a large number of unfused myosin-expressing myoblasts are present by late stage 13 (compare Figures 1A and 1B, 1C; Figure 2A and 2B). These unfused founder cells maintain the ability to differentiate and form elongated mononucleated myocytes (Figure 1B). All skeletal muscles appear to be affected in *ants* mutant embryos. On the other hand, the visceral muscles exhibit only minor defects in the first midgut constriction, and the dorsal vessel appears to be normal (data not shown). The *ants* mutation, therefore, is highly specific to the somatic musculature.

### *ants* Is Required for Myoblast Fusion

In principle, the large number of unfused myoblasts in *ants* mutant embryos could be due to a specific defect in myoblast fusion or to the secondary consequences of defects in myoblast fate determination or other developmental processes (Bate, 1993). To distinguish among these possibilities, we examined several developmental processes that might indirectly affect muscle differentiation. These include the specification of the muscle founder cells and myoblasts, the pattern of innervation by motorneurons, and differentiation of the epidermis. The specification of the muscle founder cells in *ants* mutant embryos was assessed by the expression of *Krüppel* (*Kr*). It has been shown that, 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 (Ruiz-Gómez et al., 1997). Thus, KR staining appeared as clusters in wild-type embryos (Figure 2C). As shown in Figure 2D, KR was expressed in its characteristic positions in *ants* mutant embryos, suggesting that the fate of these founder cells was properly established. The number of KR-positive nuclei was reduced in *ants* mutant embryos, since founder cells failed to recruit surrounding fusion-competent cells into the clusters. We also examined the expression of *Dmef2*, a gene involved in muscle differentiation, which marks all somatic, visceral, and cardiac myoblasts (Nguyen et al., 1994; Lilly et al., 1995; Bour et al., 1995) (Figure 2E). The number of DMEF2-expressing cells in *ants* mutant embryos was comparable to that in wild-type embryos, suggesting that the mutant myoblasts initiated the differentiation program despite a block at the myo-

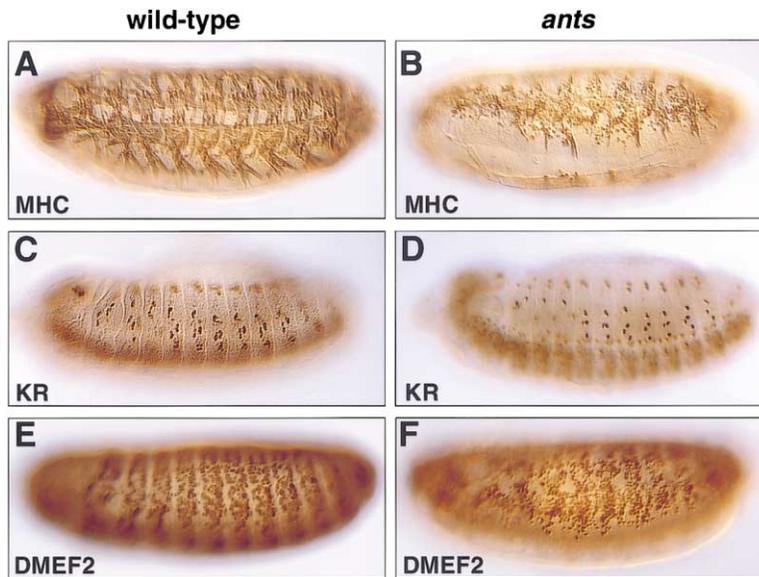


Figure 2. Founder Cell Specification and Differentiation of Muscle Precursors in *ants* Mutant Embryos

Wild-type (A, C, and E) and *ants*<sup>T627</sup> mutant embryos (B, D, and F) were stained with anti-MHC (A and B), anti-Krüppel (KR) (C and D), and anti-DMEF2 (E and F) antibodies. Embryos are oriented with dorsal up and anterior to the left.

(A and B) Ventrolateral view of stage 15 wild-type and *ants*<sup>T627</sup> embryos showing MHC expression. Note that MHC is expressed in unfused myoblasts in the *ants* mutant embryo (B). (C and D) Lateral view of stage 13 wild-type and *ants*<sup>T627</sup> 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 (C). In the *ants* mutant embryo (D), KR is expressed in isolated, instead of clusters of, nuclei due to lack of fusion.

(E and F) Lateral view of stage 14 embryos showing similar number of DMEF2-expressing myoblasts in wild-type and *ants*<sup>T627</sup> mutant embryos.

blast fusion stage (Figures 2E and 2F). Furthermore, the pattern of innervation by motoneurons, revealed by the expression of Fasciclin II (Grenningloh et al., 1991), and the differentiation of epidermis, revealed by cuticle preparation and Fasciclin III expression (Patel et al., 1987), were normal in *ants* mutant embryos (data not shown). Thus, we conclude that the unfused myoblast phenotype in *ants* mutant embryos is likely due to a specific defect in myoblast fusion.

Previous analyses have shown that myoblast fusion arrests at distinctive stages in different mutants (Dobberstein et al., 1997; Paululat et al., 1999; Ruiz-Gómez et al., 2000). In *duf* mutant embryos, fusion-competent myoblasts extend filopodia at random orientations and are not attracted by founder cells (Ruiz-Gómez et al., 2000). In *blow* and *singles-bar* mutant embryos, on the other hand, fusion-competent myoblasts form clusters around the founder cells and extend filopodia toward their fusion targets, indicating that the fusion process arrests at a stage later than the initial attraction (Dobberstein et al., 1997; Ruiz-Gómez et al., 2000). Detailed analysis of *ants* mutant embryos revealed that fusion-competent myoblasts extend filopodia toward their fusion targets (Figure 1C). This observation suggests that *ants* is dispensable for the initial attraction between founder cells and fusion-competent myoblasts and instead functions at a later stage in the fusion process.

#### Molecular Cloning of *ants*

*ants* was localized between *h* and *th* based on recombination mapping. One deficiency, *Df(3L)vin4* (68B1-2; 68F3-4), did not complement any of the four *ants* alleles, whereas another two deficiencies, *Df(3L)vin3* (68C5-6; 68E3-4) and *Df(3L)vin2* (67F2; 68D6), complemented the *ants* alleles. Thus, *ants* was localized to chromosomal region 68E-F. Embryos transheterozygous for any of the four *ants* alleles and deficiency *Df(3L)vin4* exhibited the

same myoblast fusion phenotype as the homozygous mutants, suggesting that these *ants* alleles behave as null alleles.

From a collection of lethal P element insertions in the 68E-F region, we identified two lines, *l(3)08232* and *A490.2M3*, that failed to complement the lethality of *ants*. Both P element lines showed a myoblast fusion phenotype similar to that of the EMS *ants* alleles. We mapped the P element insertion site of *A490.2M3* to 997 bp upstream of the transcriptional start site of a predicted transcript CG12277 (Figure 3A) (Adams et al., 2000). The P element insertion site of *l(3)08232* has been mapped by the Berkeley *Drosophila* Genome Project to 289 bp upstream of the transcriptional start site of another predicted transcript CG6793, which is located within a 26 kb intron of CG12277 (Figure 3A) (Adams et al., 2000). DNA sequencing of all four *ants* mutants revealed no molecular lesion in CG6793. Thus, we focused our analysis on CG12277 as a candidate gene for *ants*. Searching the *Drosophila* EST database with the predicted transcript of CG12277 identified one cDNA clone, GH15583, whose 5' end matched CG12277. The 3' end of GH15583, however, did not match any sequence within the predicted CG12277; rather, it matched CG5679, another predicted transcript approximately 15 kb 3' of CG12277 (Figure 3A). This observation suggested that CG12277 and CG5679 might represent one transcript.

Several experiments were performed to further examine the possibility that CG12277 and CG5679 were derived from a single gene. First, we sequenced the entire length of GH15583 and found that the 5.1 kb cDNA indeed spanned both predicted transcripts and included most of the predicted exons of both transcripts (Figure 3A). This cDNA clone is predicted to contain 5' UTR and 3' UTR (Figure 3A), suggesting that it represents a full-length or near full-length cDNA clone. Consistent with

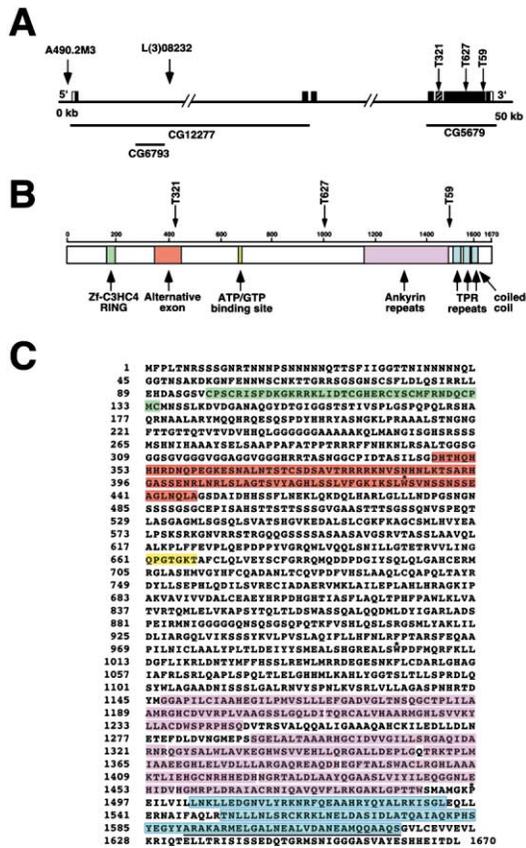


Figure 3. Molecular Characterization of *ants*

(A) Genomic organization of the *ants* gene. Black boxes represent exons, and white boxes represent untranslated regions (UTRs). The alternative exon specific to *ants iso2* is indicated by a striped box. The insertion sites of the two P element alleles, as well as the positions of the point mutations in three *ants* EMS alleles, are indicated by arrows. The extent of the three predicted transcripts (CG12277, CG6793, and CG5679) in this region, as published in the Berkeley *Drosophila* Genome Project, is also indicated.

(B) Schematic structure of ANTS ISO2 protein. ANTS ISO2 is predicted to encode a 1670 amino acid polypeptide containing a RING finger, an ATP/GTP binding motif (P loop), 9 ankyrin repeats, 3 TPR repeats, and a coiled-coil domain.

(C) Amino acid sequence of ANTS ISO2. The alternative exon (amino acids 347–447) is shaded in red. The RING finger (green), ATP/GTP binding site (yellow), ankyrin repeats (purple), and the TPR repeats (blue) are indicated. The coiled-coil domain that overlaps with the third TPR repeat is underlined. Asterisks mark the positions of point mutations in *ants*<sup>T627</sup>, *ants*<sup>T321</sup>, and *ants*<sup>T59</sup>.

that, Northern blot analysis with GH15583 revealed a cluster of transcripts of approximately 5–6 kb that are most abundant in embryos, with decreasing levels in larvae and adults (data not shown). To investigate the possibility that differential splicing contributed to the multiple signals seen in the Northern analysis, we performed RT-PCR using several primer pairs derived from GH15583. This experiment revealed an mRNA species that contained an additional 303 bp exon (Figure 3A). Thus, the CG12277/CG5679 gene from which GH15583 is derived generates at least two differentially spliced isoforms, isoform-1 (iso1) of 5.1 kb that is identical to GH15583, and isoform-2 (iso2) of 5.4 kb. ISO1 has an

ORF of 1569 amino acids, while ISO2 contains an ORF of 1670 amino acids. These isoforms only differ in the inclusion of an additional 101 amino acids in ISO2.

We then examined whether mutation in the CG12277/CG5679 gene was responsible for the *ants* phenotype. Genomic DNA from homozygous *ants* mutant embryos was sequenced using primers spanning the CG12277/CG5679 locus. *ants*<sup>T627</sup> contained a G to A point mutation that would change amino acid 1002 of ISO2 (or aa 901 of ISO1) from Trp to a stop codon. *ants*<sup>T321</sup> contained a G to A mutation that changed Trp-431 of ISO2 to a stop codon. Interestingly, this residue is located within the 101 amino acid exon that is present in ISO2 but absent from ISO1, suggesting that ISO2 is essential for the fusion process. *ants*<sup>T59</sup> contained a C to T mutation that changed amino acid 1496 of ISO2 (or aa 1395 of ISO1) from Pro to Ser. No molecular lesion was detected in the coding region of the *ants*<sup>T192</sup> allele, suggesting that the *ants*<sup>T192</sup> mutation may have disrupted the regulatory sequence of *ants*. Taken together, the molecular lesions in *ants* mutants strongly suggest that the CG12277/CG5679 gene corresponds to *ants* and that the 5.4 kb ISO2 is indispensable for myoblast fusion.

#### Domain Structures of the ANTS Protein

The predicted primary sequence of the ANTS protein contains several domains that are known to mediate protein-protein interactions (Figures 3B and 3C). In the carboxy-terminal region, nine ankyrin repeats are followed by three TPR repeats and a coiled-coil domain. In addition, the ANTS protein contains a C3HC4 RING finger near its amino-terminal region, along with a putative ATP/GTP binding site (P loop). Ankyrin repeats have been shown to interact with a variety of proteins, including the cytoplasmic domain of adhesion molecules, ion channels, and cytoskeleton proteins (for review, see Rubtsov and Lopina, 2000). TPR repeats have also been implicated in mediating a range of protein-protein interactions, including interactions with homeodomain and SH2 domains (for review, see Blatch and Lässle, 1999). The T321 and T627 mutations are predicted to truncate the ANTS protein at amino acid 431 and 1002, respectively, thus removing all the ankyrin repeats, the TPR repeats, and the coiled-coil domain, implicating these domains in the function of ANTS. The T59 mutation, which changed amino acid 1496 from Pro to Ser, is located carboxy-terminal of the ankyrin repeats and amino-terminal of the TPR repeat and the coiled-coil domain. It is not clear at present how this mutation might affect the normal function of the ANTS protein.

#### Expression Pattern of *ants*

The embryonic expression pattern of *ants* was examined by in situ hybridization and antibody staining. *ants* expression is initiated at embryonic stage 11 in the progenitors of the visceral, somatic, and pharyngeal muscles (Figure 4A). As germ band shortening proceeds, the visceral mesodermal expression of *ants* gradually decreases, while the somatic mesodermal expression persists until stage 14 (Figures 4C and 4E). By stage 15, *ants* is no longer expressed in the mesoderm. Instead, weak expression of *ants* can be detected in the muscle attachment sites along the segment borders (data not

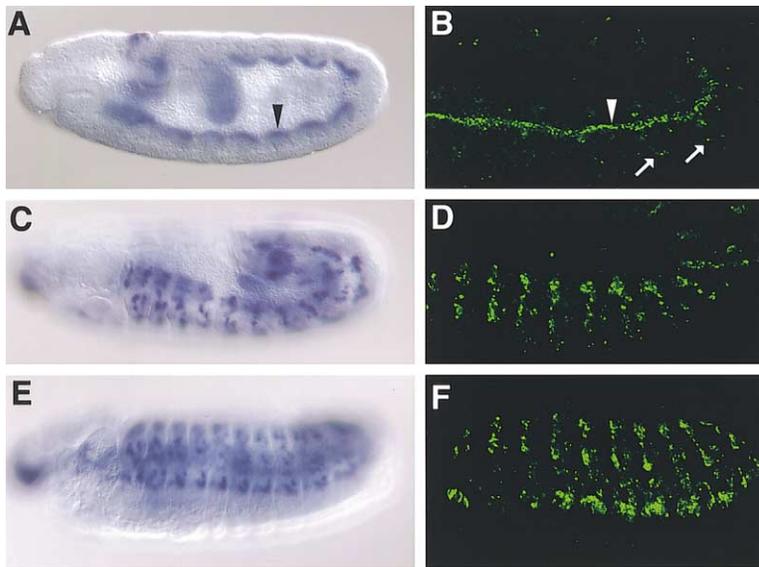


Figure 4. Expression Pattern of *ants* during Embryonic Development

(A, C, and E) Localization of *ants* transcript in wild-type embryos detected by RNA in situ hybridization. (B, D, and F) Confocal images of ANTS protein distribution (green). The images are the projection of ten consecutive Z sections in 2  $\mu\text{m}$  intervals. Lateral views of embryos are shown; anterior is to the left. The ANTS protein exhibits a similar expression pattern to that of the *ants* transcript. (A) and (B) show early stage 12. *ants* is expressed in the somatic (arrow) and visceral (arrowhead) mesoderm. The somatic mesoderm is out of focus in (A). (C) and (D) show early stage 13, (E) and (F), stage 14. *ants* expression is maintained at a high level (until early stage 15) in the somatic mesoderm, which coincides with the progress of myoblast fusion.

shown). Antibody staining revealed an ANTS protein expression pattern (Figures 4B, 4D, and 4F) similar to that of the transcript. It has been demonstrated that myoblast fusion begins at the onset of germ band retraction and is completed at the end of germ band shortening (Bate, 1990). Therefore, the temporal expression of *ants* in the somatic mesoderm coincides exactly with the fusion process and further implicates *ants* in myoblast fusion.

#### ANTS Is a Founder Cell-Specific Cytoplasmic Protein

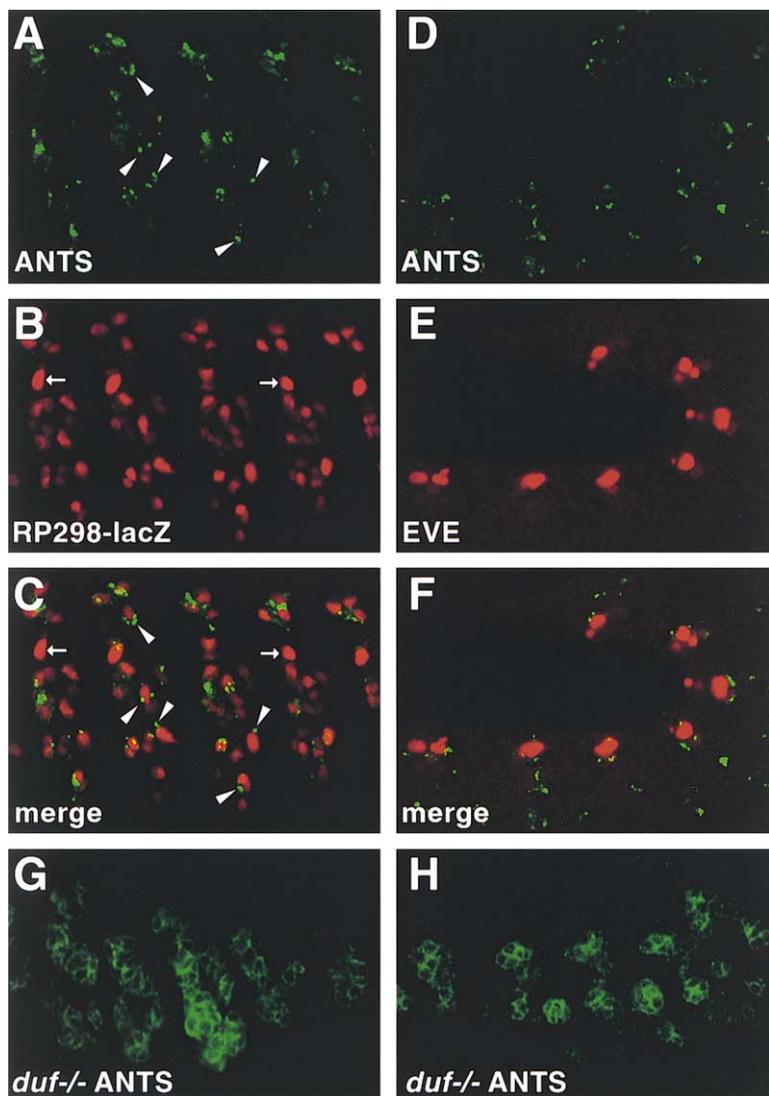
In order to gain further insights into the function of *ants* during myoblast fusion, we examined whether ANTS is present in founder cells or fusion-competent myoblasts. An antibody double-labeling experiment was performed with anti-ANTS and anti- $\beta$ -galactosidase ( $\beta$ -gal) antibodies using the *rp298* enhancer trap line, which carries a P element insertion in the 5' promoter of the *duf* gene (Nose et al., 1998; Ruiz-Gómez et al., 2000). Confocal microscopy demonstrated that ANTS was localized to the *lacZ*-expressing founder cells (Figures 5A–5C). Another founder cell-specific marker, *even-skipped* (*eve*) (Frasch et al., 1987), was also found to localize to the same cells as ANTS (Figures 5D–5F). Interestingly, ANTS is a cytoplasmic protein that aggregates to discrete foci (Figure 5). The aggregated appearance of ANTS staining is reminiscent of that of SNS, the transmembrane receptor of fusion-competent myoblasts, which is localized to discrete sites associated with the cell membrane as fusion progresses (Bour et al., 2000).

#### ANTS Interacts with DUF and MBC

The aggregation of ANTS in distinctive cytoplasmic locations in founder cells, and the presence of multiple protein-protein interaction motifs in the ANTS protein prompted us to examine if ANTS plays a role during myoblast fusion by mediating interactions between molecules in the myoblast fusion pathway(s). To test whether ANTS interacts with other fusion molecules, we

performed coimmunoprecipitation assays in *Drosophila* S2 cells using MYC-tagged ANTS and other fusion proteins, including BLOW, DUF, MBC, and SNS, tagged with the V5-epitope at their carboxyl termini. As shown in Figure 6, ANTS interacted with the founder cell receptor DUF but not the fusion-competent cell receptor SNS, despite the high homology shared by these two molecules. This specific interaction between ANTS and DUF is consistent with the founder cell-specific expression of ANTS. We noticed that a cleaved form of DUF was generated when full-length DUF was expressed in S2 cells (Figure 6B, arrowheads). This form migrated slightly slower than the DUF cytoplasmic domain alone (Figure 6B; see below), suggesting that it is likely to contain both the transmembrane and the cytoplasmic domains. Interestingly, this cleaved form also associated with ANTS (Figure 6B). When the DUF cytoplasmic domain alone was tested, however, no interaction was detected (Figure 6B). These results suggest that the transmembrane domain of DUF is required for its interaction with ANTS. In addition, protein-protein interaction was detected between an amino-terminal fragment of MBC and ANTS, while no interaction was detected between BLOW and ANTS (Figure 6B). We were unable to test the interactions between full-length MBC and ANTS, since the full-length MBC was not expressed at a detectable level.

To locate the specific domain(s) of ANTS that are required for its interaction with DUF, we made a carboxy-terminal deletion (ANTS- $\Delta\text{C}$ ) that truncated the conserved region between *Drosophila* ANTS and its mouse orthologs (see below). This deletion construct was tested for its ability to associate with DUF in coimmunoprecipitation experiments. As shown in Figure 6, no interaction between the truncated ANTS protein and DUF was detected, suggesting that the conserved region of ANTS is required for its interaction with DUF. This conclusion is consistent with the genetic mutants, since the *ants*<sup>T321</sup> allele produces carboxy-terminal-truncated protein that deletes the entire conserved region.



**Figure 5. Subcellular Localization of ANTS to Discrete Cytoplasmic Foci in Founder Cells and Its Altered Localization in *duf* Mutant Embryos**  
(A–C) Confocal images of a stage 13 embryo carrying *rp298-lacZ* and double-labeled with anti-ANTS (green) and anti- $\beta$ -gal (red) antibodies. Three images are shown, one of ANTS staining (A), one of RP298-lacZ staining (B), and one of superimposed ANTS and RP298-lacZ staining (C). ANTS staining appears as discrete foci (arrowheads pointing to a few foci adjacent to founder cell nuclei. Note that ANTS staining in some founder cells (arrows) was not in the same focal plane as shown in (A)–(C), but was detected in other focal planes (data not shown). In addition, in all cases examined, ANTS expression was associated with founder cells.  
(D–F) Wild-type stage 11 embryos double-labeled with anti-ANTS (green) and anti-EVE (red) antibodies. Three images are shown, one of ANTS staining (D), one of EVE staining (E), and one of superimposed ANTS and EVE staining (F). EVE is only expressed in a subset of founder cells, and ANTS is expressed in discrete dots associated with all of the EVE-positive cells.  
(G and H) *duf* mutant embryos stained with anti-ANTS antibody. Confocal images of a stage 13 (G) or a stage 11 (H) embryo are shown. Note that ANTS staining is distributed throughout the cytoplasm at the peripheral membrane region and appears as rings that outline the founder cells, rather than as localized discrete foci in the cytoplasm as seen in wild-type embryos. Compare ANTS staining in (G) and (H) with that in (A), (C), (D), and (F).

### The Subcellular Localization of ANTS in *duf* Mutant Embryos

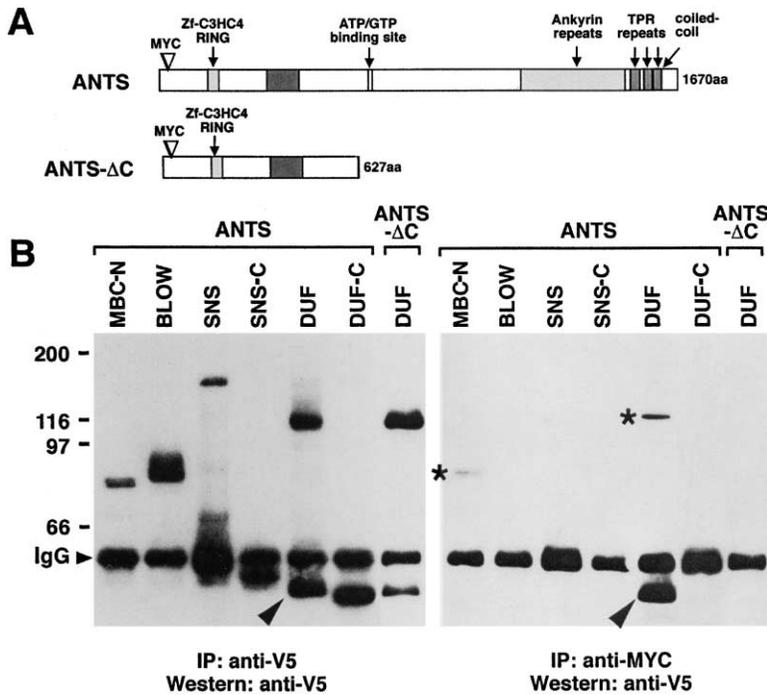
The interaction between ANTS and DUF, together with the subcellular aggregation of the ANTS protein, suggested that ANTS is likely to colocalize with DUF during myoblast fusion. Because of the lack of DUF antibody, we were unable to test this hypothesis directly. However, if DUF is involved in recruiting ANTS to specific subcellular locations during fusion, one would expect a change in the pattern of ANTS localization in *duf* mutant embryos. Examination of ANTS protein in *duf* mutant embryos showed this to be the case. Instead of localizing to discrete sites in the cytoplasm, ANTS protein is distributed throughout the cytoplasm at the peripheral membrane region and appears as rings that outline the founder cells in the *duf* mutant embryo (Figures 5G and 5H).

### A Mouse Ortholog of *ants* Is Expressed in the Embryonic Mesoderm

Database searches identified two predicted mouse proteins, mCP20090 and mCP14686 (Celera mouse genome annotation), and two human ESTs, KIAA1728 and

KIAA1636, which encode apparent orthologs of *ants* (Figure 7A). The human EST KIAA1728 (1644 amino acids) is 591 amino acids longer at its carboxyl terminus than its mouse homolog, mCP20090 (1051 amino acids), suggesting that the predicted mouse protein is missing a portion of its carboxy-terminal sequence.

To investigate whether the mammalian orthologs could also be involved in skeletal muscle development, we examined the expression of the mouse orthologs in the developing embryonic mesoderm by in situ hybridization. For simplicity, the mouse orthologs are referred to as *mants1* (mCP20090) and *mants2* (mCP14686). As shown in Figure 7B, *mants1* is expressed in a broad range of the embryonic mesodermal tissues, including the limb buds and the somites at embryonic day 11.5, coincident with the time period when myoblast fusion occurs (Hauschka, 1994). *Mants1* expression dramatically decreases at E13.5, when muscle differentiation is almost completed (data not shown). Northern blot of adult tissues showed that *mants1* is not detectable in adult skeletal muscle (data not shown). Thus, *mants1* is expressed during a short time window when myoblast fusion takes place. The expression pattern of *mants2*,



**Figure 6.** ANTS Interacts with DUF and MBC  
(A) Schematic structures of the MYC-tagged full-length ANTS and ANTS- $\Delta$ C, a carboxy-terminal deletion construct lacking the region conserved between *Drosophila* and mouse. Triangles point to the positions at which the MYC-epitope was inserted.  
(B) S2 cells coexpressing MYC-tagged ANTS (or ANTS- $\Delta$ C) and each of the other fusion proteins (tagged with V5-epitope at their carboxyl termini) were lysed, and total cell lysate was immunoprecipitated (IP) with anti-V5 (left panel) and anti-MYC (right panel) antibodies, respectively, and probed with anti-V5 on a Western blot. BLOW, SNS, and DUF represent full-length proteins. MBC-N is an amino-terminal fragment of MBC. SNS-C and DUF-C are the cytoplasmic domains of SNS and DUF, respectively. The left panel shows the input of the V5-tagged proteins. Note the presence of a cleaved form of DUF (arrowhead) in the ANTS/DUF lane that migrated slightly slower than DUF-C. The right panel shows that MBC-N (asterisk), full-length DUF (asterisk), and the cleaved form of DUF (arrowhead) were coimmunoprecipitated with ANTS, while BLOW and SNS were not. Molecular size markers are shown at the left.

on the other hand, is completely different from that of *mants1*. While *mants1* expression is absent from the neural tube and dorsal root ganglia in the E11.5 embryo, *mants2* is expressed strongly in these neural tissues. The neural expression of *mants2* persists into adult stages (data not shown). The transient expression of *mants1* in mouse embryonic tissue is consistent with the transient expression of *ants* during myoblast fusion in *Drosophila* embryos and suggests that *mants1* could play a role in skeletal muscle differentiation.

### Discussion

Myoblast fusion is a multistep process involving cell recognition, adhesion, alignment, and membrane fusion. Recent studies in *Drosophila* are beginning to reveal the components of a signaling pathway employed in the fusion process. Two transmembrane receptors, DUF and SNS, are implicated in cell recognition, whereas the cytoplasmic protein MBC has been implicated in mediating changes in the cytoskeleton. It is not clear whether or how the known fusion molecules interact with each other during the fusion process. In addition, given the multistep nature of the fusion process, it is likely that additional components of the pathway(s) remain to be identified. In this study, we present the identification and characterization of a new molecule involved in myoblast fusion. ANTS is a founder cell-specific cytoplasmic protein that interacts with both DUF and MBC. Thus, ANTS could serve as a linker molecule that relays essential signals from a membrane receptor to changes in the cytoskeleton of founder cells.

### ANTS Is an Ankyrin Repeat-Containing Protein that Interacts with DUF and MBC

*ants* encodes a molecule with multiple protein-protein interaction motifs, including nine ankyrin repeats, three

TPR repeats, a coiled-coil domain, and a RING finger. Ankyrins are known to function as linkers between integral membrane proteins and the spectrin-based cytoskeleton (Rubtsov and Lopina, 2000). Ankyrin proteins contain three domains, including a membrane binding domain at the amino terminus, a central spectrin binding domain, and a carboxy-terminal regulatory domain. The membrane binding domain, which contains multiple ankyrin repeats, binds to the cytoplasmic domains of specific integral membrane proteins including adhesion molecules. ANTS is not a conventional ankyrin protein, since its ankyrin repeats are located at the carboxy-terminal region and it lacks the central spectrin binding domain. Nevertheless, ANTS can associate with the founder cell receptor DUF and the cytoplasmic protein MBC. These interactions are specific, since ANTS does not interact with SNS, another Ig domain-containing receptor that is localized to fusion-competent myoblasts, nor does ANTS interact with BLOW, another cytoplasmic protein. Our studies further suggest that the conserved regions between ANTS and its vertebrate orthologs, including the ankyrin repeats, are required for ANTS' interaction with DUF, since a deletion construct lacking the conserved domains does not associate with DUF. That an *ants* allele (*ants*<sup>T321</sup>) that deletes the conserved region behaves as a null mutation is consistent with this region being important for the function of ANTS in vivo. Our preliminary results indicate that MBC maintains the ability to interact with an ANTS protein lacking the conserved carboxy-terminal region, suggesting that the amino-terminal domain of ANTS is likely to interact with MBC (E.H.C. and E.N.O., unpublished data).

### ANTS Is a Cytoplasmic Protein Localized in Discrete Domains in Founder Cells

Our antibody staining showed that ANTS is a cytoplasmic protein. Two other fusion molecules, MBC and

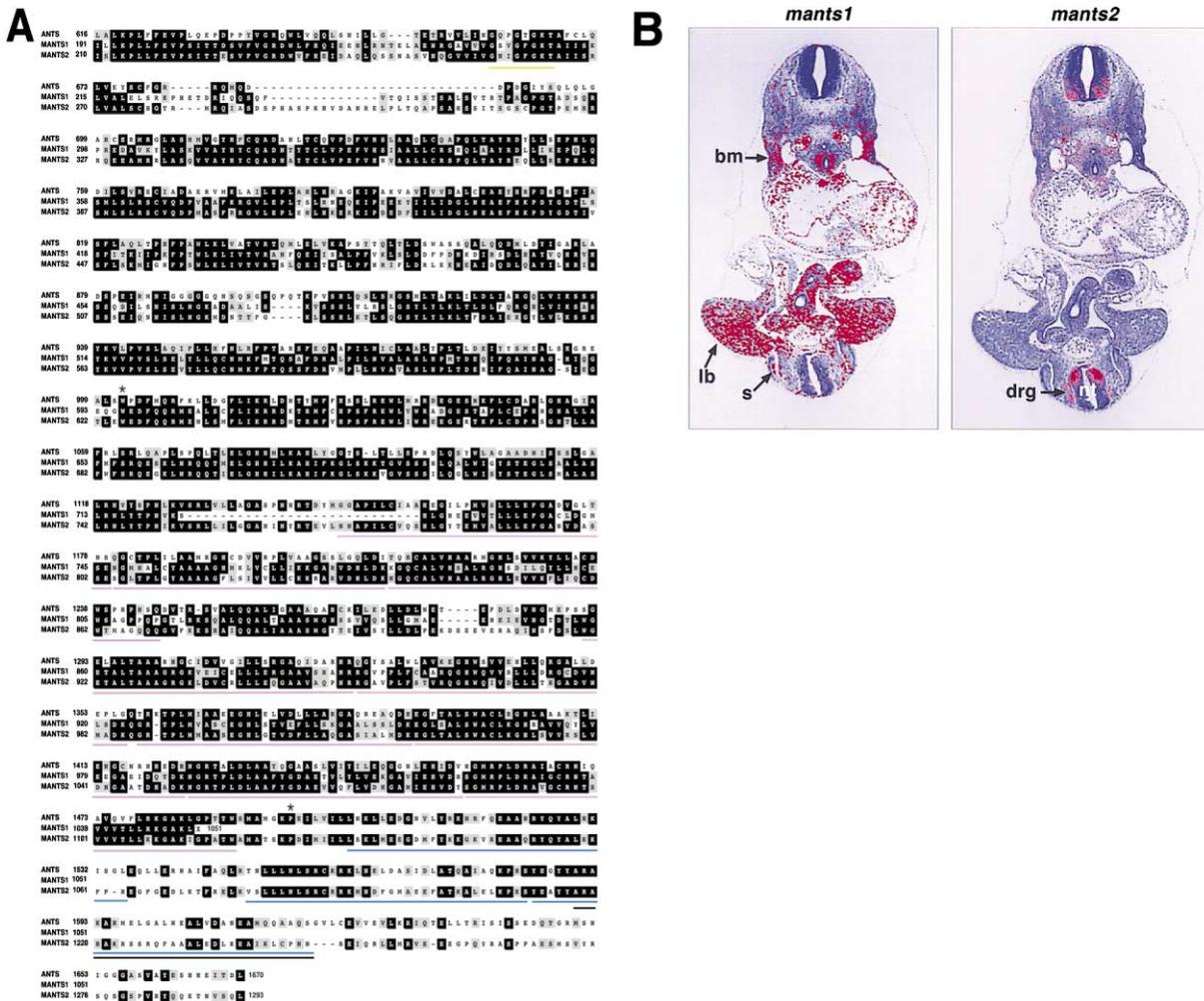


Figure 7. Sequences of Two ANTS Orthologs and Their Expression Patterns in E11.5 Mouse Embryos

(A) Alignment of ANTS with two mouse orthologs, mCP20090 (MANTS1) and mCP14686 (MANTS2). The conserved P loop (underlined in yellow), ankyrin repeats (underlined in purple), TPR repeats (underlined in blue), and coiled-coil domain (underlined in black) are shown. Mutations in *ants*<sup>759</sup> and *ants*<sup>7627</sup> are indicated by asterisks.

(B) *mants1* is expressed in a broad range of mesodermal structures. Its expression in somites (s), limb bud (lb), and cells that will give rise to the body wall muscle (bm) is marked. *mants2* is expressed predominantly in the neural tube (nt) and dorsal root ganglia (drg).

BLOW, are also expressed in the cytoplasm (Doberstein et al., 1997; Erickson et al., 1997). However, the localization of ANTS is distinct from that of MBC and BLOW. While MBC and BLOW are expressed in both founder cells and fusion-competent myoblasts, ANTS is only expressed in founder cells. In addition, while MBC and BLOW are expressed throughout the cytoplasm of myoblasts, ANTS is localized in discrete domains in the cytoplasm. These results, together with the protein-protein interaction between ANTS and DUF, raise the possibility that the ANTS localization domains might correlate with the sites of cell recognition and adhesion between founder cells and fusion-competent myoblasts. The subcellular structures in which ANTS is localized and how these domains might be related to the expression of DUF on the founder cell membrane remain to be determined. While the lack of DUF antibody prevents the examination of the DUF protein expression pattern on the founder cell membrane and the relative

localization of DUF and ANTS, the SNS protein has been shown to be clustered in discrete regions on the membrane of fusion-competent cells (Bour et al., 2000). It is conceivable that DUF may also be localized to specific membrane regions in founder cells during the fusion process. However, we cannot rule out the possibility that there is an excessive amount of DUF on the founder cell membrane such that no localization of DUF is necessary during cell recognition and cell adhesion. Nevertheless, the altered ANTS localization in *duf* mutant embryos supports the hypothesis that DUF is required to localize ANTS to specific subcellular foci, presumably through the physical interaction between the two proteins.

#### The Role of *ants* in Myoblast Fusion

Myoblast fusion requires not only the recognition and adhesion between founder cells and fusion-competent cells, but also subsequent cytoskeletal rearrangements

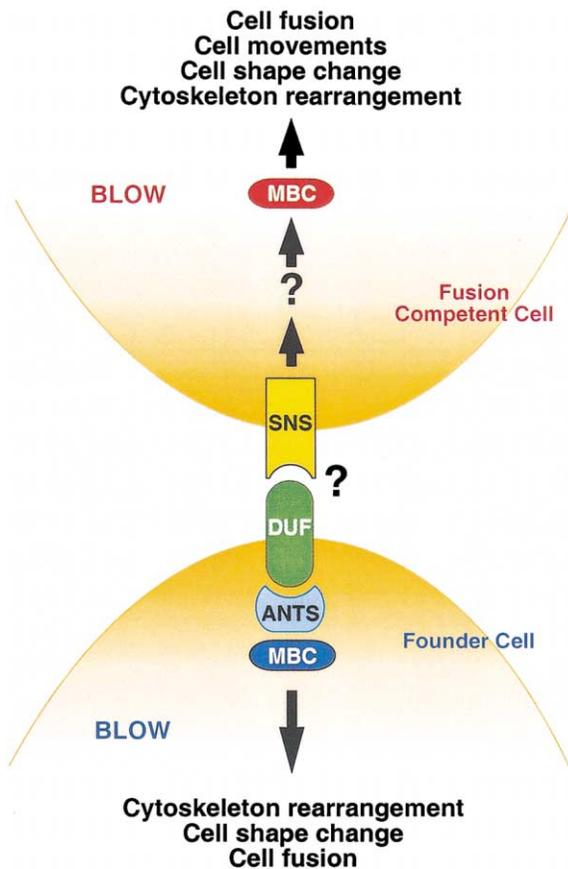


Figure 8. A Model of the Function of ANTS in Myoblast Fusion  
See text for details.

that lead to the proper alignment of the two populations of cells (Doberstein et al., 1997). Previous studies on the founder cell-specific receptor DUF have shown that it acts as an attractant for fusion-competent cells (Ruiz-Gómez et al., 2000). Although *duf* is necessary for myoblast fusion, it is not sufficient, since ectopic expression of *duf* in fusion-competent cells did not result in fusion among this population of myoblasts (Ruiz-Gómez et al., 2000). Based on this observation, it was suggested that besides *duf*, there must exist at least one additional protein that is present in founder cells but absent from fusion-competent myoblasts. This protein could interact with the intracellular domain of DUF to initiate fusion (Ruiz-Gómez et al., 2000). Our results suggest that ANTS may represent such a molecule. First, ANTS is expressed in founder cells just before and during the fusion process. Second, ANTS physically interacts with the cytoplasmic domain of DUF. Third, the ANTS protein is localized in discrete regions in the cytoplasm of founder cells during the fusion process, and the specific localization of ANTS is altered in *duf* mutant embryos, consistent with the possible interaction with a localized membrane receptor during the fusion process.

Based on these observations and the interaction between ANTS and MBC, we propose the following sequence of events during myoblast fusion (Figure 8). First, DUF acts as an attractant for fusion-competent myoblasts. Through either direct or indirect interaction(s)

between DUF and SNS, fusion-competent myoblasts recognize and adhere with founder cells. In this process, SNS is localized to discrete sites in the membrane of fusion-competent myoblasts, presumably sites of cell adhesion. It is possible that DUF is also localized to discrete domains in the membrane of the founder cells. Next, within the founder cells, through interaction(s) between the cytoplasmic domain of DUF and ANTS, ANTS is recruited to discrete cytoplasmic domains close to the membrane. Meanwhile, interaction between ANTS and MBC, and perhaps additional cytoskeleton-associated molecules, leads to changes in the cytoskeleton that are necessary for the proper alignment of founder cells with fusion-competent cells. This model predicts that in *ants* mutant embryos, despite a block of cell alignment, which requires the transmission of signals from DUF to the cytoskeleton, cell recognition and adhesion should take place normally. This is indeed what we observed. In *ants* mutant embryos, fusion-competent myoblasts extend filopodia toward their fusion targets (Figure 1C). Such phenotypes are not observed in *duf* mutant embryos in which fusion is blocked at the cell recognition step (Ruiz-Gómez et al., 2000). Taken together, we favor the model that ANTS acts as a linker molecule that relays signals from the membrane receptor DUF to changes in the cytoskeleton in the founder cells.

#### *Drosophila* and Vertebrate Myoblast Fusion

Electron microscopic studies have revealed that the cellular processes of myoblast fusion, including cell adhesion, alignment, and membrane fusion, are conserved between *Drosophila* and vertebrates (Wakelam, 1985; Knudsen, 1992; Doberstein et al., 1997). Given the conservation of numerous signaling pathways between *Drosophila* and vertebrates, it is possible that vertebrate homologs of genes required for *Drosophila* myoblast fusion might play similar roles in skeletal muscle development. However, none of the myoblast fusion genes identified in *Drosophila* so far have been implicated in a similar role in vertebrate skeletal muscle development. For example, the closest vertebrate homolog of DUF and SNS is the human Nephrin protein, which is essential for kidney development (Kestila et al., 1998; Lenkkeri et al., 1999). The vertebrate homolog of MBC, DOCK180, interacts with focal adhesion molecules and seems to be a general factor that regulates cytoskeletal events (Hasegawa et al., 1994). Our studies of two mouse orthologs of *ants* suggest that one of them, *mants1*, could be involved in skeletal muscle development in vertebrates. The temporal expression pattern of *mants1* in the developing mouse embryo is reminiscent of *ants* expression in the *Drosophila* embryo. *mants1* expression coincides with the early stages of mesodermal development, and its expression is dramatically reduced after skeletal muscle formation. The transient expression of *mants1* in the mesoderm is consistent with a potential role in early skeletal muscle development including myoblast fusion. Interestingly, *mants1* is also expressed at the time of fusion in the C2 myoblast cell line (E.H.C. and E.N.O., unpublished data). However, it should be pointed out that the expression of *mants1* in the mouse embryo is not solely restricted to skeletal muscle precursors but rather is more broadly distributed

throughout the mesoderm at E11.5. Obviously, further studies will be required to confirm if *mants1* indeed plays a role in myoblast fusion in vertebrates as does *ants* in *Drosophila*.

## Experimental Procedures

### Genetics

Four *ants* mutant alleles, *ants*<sup>759</sup>, *ants*<sup>7192</sup>, *ants*<sup>7321</sup>, and *ants*<sup>7627</sup>, were isolated in a genetic screen for myoblast fusion mutants (E.H.C. and E.N.O., unpublished data). The mutant strains carry an *MHC-tauGFP* transgene on the X chromosome and an *armadillo-GFP* on their TM3 balancer. The *ru h th st cu sr e ca* chromosome was used to meiotically map *ants* between *h* and *th*. These mutants were further mapped by overlapping deficiencies and tested for complementation with P element insertion lines.

*rp298-lacZ*, obtained from Akinao Nose, was used as a founder cell-specific marker. *Df(1)w<sup>67k30</sup>*, a null allele of *duf*, was used (Ruiz-Gómez et al., 2000).

### Molecular Biology

EST clone GH15883 was obtained from Research Genetics. To sequence *ants* mutants, homozygous mutant embryos were selected by their lack of *armadillo-GFP* that was carried on the balancer chromosome. Genomic DNA was prepared from the mutant embryos. PCR products encompassing the candidate gene were sequenced to determine if they contained specific mutations. When a mutation was discovered, PCR and sequencing were repeated to confirm that the mutation was not due to PCR error. The insertion site of the *A490.2M3* line was mapped by inverse PCR following *Sau3A* digest using a standard protocol (Berkeley *Drosophila* Genome Project).

*Drosophila* Northern analyses were performed using embryonic, larval, and adult polyA<sup>+</sup> mRNAs (Clontech). The mRNAs were electrophoresed, transferred, and probed using standard procedures. Mouse Northern analyses were performed using a mouse adult multi tissue membrane (Clontech).

RT-PCR was performed using the Superscript First-Strand Synthesis for RT-PCR kit (GIBCO-BRL). The mRNA source was embryonic polyA<sup>+</sup> mRNA (Clontech). PCR reactions were performed using Expand High Fidelity PCR system (Boehringer Mannheim) for fragments less than 3 kb and Expand Long Template PCR system (Boehringer Mannheim) for longer fragments.

### Immunohistochemistry

Antibody staining was performed as described (Patel, 1994). The primary antibodies used were as follows: rabbit anti-MHC (1:1000) (Kiehart and Feghali, 1986), 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), mouse anti-EVE (1:10) (Frasch et al., 1987), rabbit anti- $\beta$ -gal (1:1500) (Cappel), and mouse anti- $\beta$ -gal (1:2000) (Promega). A polyclonal rabbit anti-ANTS antiserum was generated using a carboxy-terminal peptide (amino acids 1656–1670) (Bio-Synthesis) and used at 1:3000. Secondary antibodies used were as follows: biotinylated goat anti-rabbit (1:300) (Vector) and Cy3 goat anti-mouse (1:300) (Jackson). Fluorescent images were collected on a LS3.95 Zeiss confocal microscope and were processed with Adobe Photoshop 5.

In situ hybridization of *Drosophila* embryos was performed using standard protocols (Tautz and Pfeifle, 1989). DIG-labeled probe was synthesized with the entire coding region of *ants iso2*.

In situ probes of *mants1* and *mants2* were synthesized as follows. Primers were designed to amplify an exon of approximately 600 bp from mouse genomic DNA. The PCR products were sequenced to ensure that correct genes were amplified. [<sup>35</sup>S]UTP in situ probes were synthesized using the MAXIScript kit (Ambion). The hybridization procedure was performed by the Pathology Core Facility at UT Southwestern Medical Center.

### S2 Tissue Culture and Coimmunoprecipitation

Constructs used for S2 cell transfection were made as follows. MYC-tagged ANTS: six copies of MYC-epitope were inserted in-frame at a KpnI site 97 bp downstream of the translation initiation site in the

pOT2 vector. The entire *ants* cDNA containing the MYC tag was then subcloned into the pAc-V5 His expression vector (Invitrogen). The carboxy-terminal deletion construct of ANTS was constructed by PCR amplification followed by subcloning into the pAc-V5 His vector. A stop codon was added to the 3' end of the PCR fragment. V5-tagged BLOW, DUF, SNS and MBC: the full-length coding regions of these genes, except for *mbc*, were amplified by PCR from cDNAs from different sources and cloned in-frame into the pAc-V5 His vector. *blow* was amplified from the EST clone SD01942. *duf* was amplified from cDNA clone HB3 (Ruiz-Gómez et al., 2000). *sns* was amplified from a full-length *sns* cDNA clone (Bour et al., 2000).

S2 cells were transfected using the Effectene transfection reagent (Qiagen). Two days after transfection, cells were lysed in immunoprecipitation buffer at 4°C as described (Gao and Pan, 2001). Immunoprecipitations were performed using rabbit anti-MYC (Santa Cruz) and mouse anti-V5 (Invitrogen) antibodies and protein G sepharose according to manufacturer's instructions. Precipitated proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and then probed with antibodies. Antibodies used to probe the Western blot membranes were mouse anti-MYC (1:2500) (Santa Cruz) and mouse anti-V5 (1:5000). The secondary antibodies used were HRP-goat anti-mouse (1:10,000) and HRP-goat anti-rabbit (1:10,000) (Bio-Rad).

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#### Accession Numbers

The GenBank accession number for the *ants* sequence reported here is AF440568 and AF440569.

