The immunoglobulin superfamily member Hbs functions redundantly with Sns in interactions between founder and fusion-competent myoblasts

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The body wall muscle of a Drosophila larva is generated by fusion between founder cells and fusion-competent myoblasts (FCMs). Initially, a founder cell recognizes and fuses with one or two FCMs to form a muscle precursor, then the developing syncitia fuses with additional FCMs to form a muscle fiber. These interactions require members of the immunoglobulin superfamily (IgSF), with Kin-of-IrreC (Kirre) and Roughest (Rst) functioning redundantly in the founder cell and Sticks-and-stones (Sns) serving as their ligand in the FCMs. Previous studies have not resolved the role of Hibris (Hbs), a paralog of Sns, suggesting that it functions as a positive regulator of myoblast fusion and as a negative regulator that antagonizes the activity of Sns. The results herein resolve this issue, demonstrating that sns and hbs function redundantly in the formation of several muscle precursors, and that loss of one copy of sns enhances the myoblast fusion phenotype of hbs mutants. We further show that excess Hbs rescues some fusion in sns mutant embryos beyond precursor formation, and as a negative regulator that antagonizes the activity of Sns. The results herein resolve this issue, demonstrating that sns and hbs function redundantly in the formation of several muscle precursors, and that loss of one copy of sns enhances the myoblast fusion phenotype of hbs mutants. We further show that excess Hbs rescues some fusion in sns mutant embryos beyond precursor formation, but show using chimeric molecules that Hbs functions less efficiently than Sns. In conjunction with a physical association between Hbs and SNS in cis, these data account for the previously observed UAS-hbs overexpression phenotypes. Lastly, we demonstrate that either an Hbs or Sns cytodomain is essential for muscle precursor formation, and signaling from IgSF members found exclusively in the founder cells is not sufficient to direct precursor formation.

KEY WORDS: Myoblast fusion, Sns, Hbs, Cell adhesion, Muscle development, Drosophila

INTRODUCTION

The body wall musculature of the Drosophila larva is comprised of a segmentally repeated array of 30 individual muscle fibers per abdominal hemisegment that develop during embryogenesis. As in vertebrates, these myofibrils are syncitial due to fusion between myoblasts. Myoblast fusion in Drosophila occurs directionally and involves two distinct populations of myoblasts: founder cells and fusion-competent myoblasts (FCMs) (Bate and Rushton, 1993). Founder myoblasts are specialized cells that dictate muscle identity, and confer on each muscle fiber unique features that include size, shape, pattern of innervation and attachment. FCMs represent a larger naïve group of cells that are lacking the complex attributes characteristic of mature muscle (Abmayr and Kocherlakota, 2005). These cells come under the influence of founder-cell-specific muscle-identity genes, becoming entrained to the myogenic program of the founder cell with which they fuse. The initial fusion event occurs between a founder cell and one or two FCMs to form a muscle precursor, whereas subsequent fusions occur between the developing syncitium and additional FCMs. In Drosophila, cell adhesion molecules of the immunoglobulin superfamily (IgSF) direct the above events, and include kin of irre (kirre; also called dumbfounded, or duf), roughest (rst; IrreC), sticks and stones (sns) and hibris (hbs) (Artero et al., 2001; Bour et al., 2000; Dworak et al., 2001; Ruiz-Gomez et al., 2000; Strunkelnberg et al., 2001). The kirre and rst loci result from gene duplication (Strunkelnberg et al., 2003) and are orthologs of syg-1 in Caenorhabditis elegans (Shen and Bargmann, 2003) and neph-1-4 in mammals (Sellin et al., 2003). Kirre is exclusive to the founder cells (Ruiz-Gomez et al., 2000), whereas Rst is present in founder cells and at least some FCMs (Strunkelnberg et al., 2001). Although no role has been identified for Rst in the FCMs, Kirre and Rst function redundantly in the founder cell (Strunkelnberg et al., 2001). Embryos lacking both kirre and rst exhibit no myoblast fusion, a defect that is rescued by mesodermal expression of either gene (Ruiz-Gomez et al., 2000; Strunkelnberg et al., 2001). The FCM-specific IgSF proteins Sns and Hbs share 48% identity (Artero et al., 2001; Bour et al., 2000; Dworak et al., 2001). Like their orthologs C. elegans syg-2 (Shen et al., 2004) and vertebrate nephrin (Kestila et al., 1998), Sns and Hbs are predicted to include nine Ig domains and one fibronectin type-III domain in their extracellular regions. Their cytoplasmic domains differ in length, corresponding to 374 amino acids and 165 amino acids, respectively. Sns is restricted to the FCMs, appears on their surface just before fusion, and is often coincident with Kirre or Rst at points of cell-cell contact (Bour et al., 2000; Galletta et al., 2004). Hbs is also restricted to the FCMs, where it declines slightly before Sns. In cells that express both proteins, Sns and Hbs co-localize at discrete points on the cell surface (Artero et al., 2001). Despite these similarities, Sns and Hbs have distinct roles from each other in the FCMs. Whereas embryos lacking sns exhibit a dramatic absence of multinucleate syncitia, embryos lacking hbs exhibit only a modest perturbation of myoblast fusion, which does not impair their survival. Moreover, although some studies have suggested that Hbs acts antagonistically to limit Sns activity (Artero et al., 2001), others suggest that Hbs acts positively to direct limited myoblast fusion in the absence of Sns (Menon et al., 2005).

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Sns appears to act as a receptor for Kirre and Rst, mediating the ability of FCMs to recognize and adhere to founder cells. Intracellular pathways downstream of these proteins then direct myoblast fusion. Downstream of Kirre is the guanine nucleotide exchange factor Schizo (Loner), which probably activates Rac1 via the GTPase Arf151F (Arf6) (Chen et al., 2003). The cytoplasmic domain of Kirre is also linked to the non-conventional guanine nucleotide exchange factor Mbc (Erickson et al., 1997) through interaction with Rolling pebbles (Rols; Antisocial, or Ants) (Chen and Olson, 2001). Whereas Kirre and Rols are exclusive to the founder cells, some of this machinery is present and may be required in both founder cells and FCMs. For example, expression of Mbc exclusively in the founder cells is insufficient to rescue the mutant phenotype (Balagopalan et al., 2006). Mbc functions in concert with Ced-12 (Elmo) to activate the small GTPases Rac1 and Rac2, which mediate a spectrum of interactions (Kocherlakota et al., 2008).

The following mutant alleles have been reported: Fly stocks requires signaling pathways that are downstream of Sns and Hbs. Than Sns, a limitation that rests primarily within its cytoplasmic embryos supports a model in which Hbs functions less efficiently which Hbs functions positively to direct myoblast fusion. The ability via SCAR (Berger et al., 2008; Richardson et al., 2007) and the of Actin 5C (F-actin) through the Arp14D/66B (Arp2/3) complex Ced-12 (Elmo) to activate the small GTPases Rac1 and Rac2, which phenotype (Balagopalan et al., 2006). Mbc functions in concert with (Berger et al., 2008; Kim et al., 2007; Massarwa et 2007) suggest that the WASp/Vrp1 complex is connected to the Sns sites of myoblast fusion and modulated by these proteins (Kesper et al., 2001). The biochemical interactions necessary for activation of the pathway are not well understood. Indeed, multiple redundant pathway are not well understood. Indeed, multiple redundant (Artero et al., 2001). Stocks described elsewhere (Cambridge, MA). The transgenes were genetically recombined into snu snslacZ (Kocherlakota et al., 2008) and UAS- snslacZ, or (Rusu-Gomez et al., 2000), rsteC (Strunkelnberg et al., 2001), hbsXba and hbsKspI (Artero et al., 2001). Stocks described elsewhere include me2Gal4 (Ranganayakulu et al., 1998), UAS-sns-HA, snslacZ and snsGal4 (Kocherlakota et al., 2008) and UAS-hbs (Artero et al., 2001). The sns460 allele was a gift from Elizabeth Chen. The sns460 hbsXba double mutant stock was generated by EMS mutagenesis of an isogenized hbs460 chromosome to generate mutations in sns (see Fig. S1 in the supplementary material). The molecular lesions in sns460 and snsD correspond to Y333X at nucleotide position 1547 and W215R at nucleotide position 1191, respectively. Transgenic stocks were generated by Genetic Services (Cambridge, MA). The transgenes were genetically recombined into snu snslacZ or snse1160 and balanced with CyO. P[tr/+ |7.2] / en1 w gen1160 from the Bloomington Stock Center.

Cloning and constructs
The following constructs have been described: pUAST-sns-HA (Kocherlakota et al., 2008), pUAST-hbs (Artero et al., 2001), pUAST-sns20-5HA (Kocherlakota et al., 2008). To generate pUAST-kirre-HA, an EcoRV-SalI fragment that includes the entire kirre-HA coding sequence from pRhM3- kirre-HA (Galletta et al., 2004) was sub-cloned into pBSK, recovered as a NotI-KpnI fragment and cloned into pUAST (Brand and Perrimon, 1993). To generate pUAST-hbsΔCD-HA an HA epitope tag followed by a stop codon and an Xbal restriction site were introduced after AA 1114 of Hbs. The pUAST-sns-V5, pUAST-sns-Flag, pUAST-hbs-HA, pRhM3-sns-V5, pRhM3-hbs-HA and pRhM3-kirre-Flag constructs were each engineered using PCR. In each, a single repeat of the epitope tag was inserted after the last codon of the corresponding open reading frame (ORF). Domain swap constructs of sns and hbs were generated by PCR using the following chimeric oligonucleotides: (5′-CAGGCACCGACA- GAAGTTGTCTACAGAGCGAGCCGA-3′) for pUAST-SETHC-HA, (5′-CATGCTGTGAGTGATTTCAATATGACAACTCGTGTGTC-3′) for pUAST-SEHTC-HA and (5′-GAATGCGCCACAGAGATGCCTAATCATACATTTGGCGACTGTC-3′) for pUAST- HESTC-HA. Chimeric sequences for SETHC-HA and SEHTC-HA were substituted for Sns in pUAST-sns (Galletta et al., 2004) using ApI and Xbal. The chimeric sequence for HESTC-HA was substituted for Hbs in pUAST-hbs (Artero et al., 2001) using BglII and Xbal. An HA epitope tag, stop codon and Xbal site were engineered to follow the last amino acid in each chimeric ORF. The entire cDNA region of all constructs was sequenced before injection. Of note, this analysis found the cytoplasmic sequence to be consistent with that published by Dworak and colleagues (Dworak et al., 2001).

Immunohistochemistry
Embryos were collected and processed as described (Erickson et al., 1997). Homozygous mutant embryos were identified by absence of β-galactosidase (β-gal) activity. Primary antibodies to myosin heavy chain (MHC) (1:1000, D. Diekhart), Even skipped (Eve, 1:1000, M. Frasch), Pterocard (Prc, 1:10, Developmental Society Hybridoma Bank), Kruppel (Kr, 1:300, East Asian Developmental Center for Segmentation Antigen at and the Institute of Genetics, Division of Developmental Genetics, Mishima, Japan) and rabbit polyclonal to Nautilus (Nau, AA 29-143, 1:100) were used in this study. Colorimetric detection was performed using biotinylated anti-mouse and anti-rabbit IgG (1:200) and the Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions. Embryos were imaged using a Zeiss Axioplan2. Fluorescent detection used Alexa-Fluor-conjugated secondary antibodies (1:200; Invitrogen, Carlsbad, CA). Stained embryos were imaged using a Zeiss LSM-510 confocal microscope and analyzed using AIM (Zeiss) and Imaris (Bitplane) software.

Statistical analysis
Nuclei were visualized in late stage 15 embryos and were manually quantitated from confocal z-series. For mutant alleles, three (Eye and Kr) or four (Nau) abdominal hemisegments were analyzed per embryo. For rescue of snsW1, hbsXba double mutants, six (Eye and Kr) abdominal hemisegments were analyzed. Data are represented as the mean number of nuclei per hemisegment ± s.e.m., where n equals the number of hemisegments. Unfused myoblasts were visualized by anti-Sns antisera or antibodies to β-gal (Cappel, MP Biomedical) in conjunction with snslacZ (Kocherlakota et al., 2008). The number of unfused myoblasts in late stage 15 embryos was quantitated using Imaris software (Bitplane), with manual editing of confocal z-series. Quantitation of unfused myoblasts assayed three abdominal hemisegments in rescues using Hbs and Sns-Hbs chimera and four abdominal hemisegments in Sns overexpression and sns, hbs genetic interaction. The mean number of unfused myoblasts is indicated ± s.e.m., where n equals the total number of embryos.

MATERIALS AND METHODS
Fly stocks
The following mutant alleles have been reported: snuXba, snsXba, Df(2R)BB1 (Bour et al., 2000), mbsXba, Df(1)w67YK30 (Ruiz-Gomez et al., 2000), rsteC (Strunkelnberg et al., 2001), hbsKspI and hbsKsp2 (Artero et al., 2001). Stocks described elsewhere include me2Gal4 (Ranganayakulu et al., 1998), UAS-sns-HA, sns lacZ and snsGal4 (Kocherlakota et al., 2008) and UAS-hbs (Artero et al., 2001). The sns460 allele was a gift from Elizabeth Chen. The sns460 hbs460 double mutant stock was generated by EMS mutagenesis of an isogenized hbs460 chromosome to generate mutations in sns (see Fig. S1 in the supplementary material). The molecular lesions in sns460 and snsD correspond to Y333X at nucleotide position 1547 and W215R at nucleotide position 1191, respectively. Transgenic stocks were generated by Genetic Services (Cambridge, MA). The transgenes were genetically recombined into snuXba or snsZf1.4 and balanced with CyO. P[tr/+ |7.2] / en1 w gen1160 from the Bloomington Stock Center.
S2 cell culture, transfection and aggregation

S2 cells were grown and transiently transfected as described (Cherbas and Cherbas, 1998). Plasmids pUAST-sns-V5, pUAST-sns-HA, pUAST-sns-Flag and pUAST-hbs were co-transfected with pWAGal4 (Ishimaru et al., 2004) as described in Results. Cells transfected with pRmHa3-sns-V5, pRmHa3-hbs-HA or pRmHa3-kirre-Flag were induced for 16 hours with 0.7 mM CuSO4. Immunoprecipitations used cells transfected with pUAST constructs grown for 24 hours after DNA removal. Aggregations were carried out as described (Beiber, 1994; Galletta et al., 2004), with the following modifications: cells were transfected separately with pRmHa3-sns-V5, pRmHa3-hbs-HA or pRmHa3-kirre-Flag and then mixed at a 1:1:1 ratio at a final concentration of 4.5 × 10^6 cells/ml. Aggregation results from three independent experiments are presented as the percentage of Sns- or Hbs-positive cells not in contact with Kirre-positive cells ± s.e.m.}

RESULTS

Hbs, but not Rst, acts positively to direct precursor formation that occurs in the absence of SNS

As a first step in addressing the fusion observed in the absence of Sns, we systematically examined the Eve-expressing founder cell for muscle DA1 in embryos with defects in myoblast fusion. At the onset of myogenesis in a wild-type embryo, Eve is detected in two pericardial cells and the closely situated founder cell for DA1 (Fig. 1A). As fusion progresses, Eve is detected in nuclei contributed by the FCMs (Fig. 1B,C). These nuclei spread out as the muscle stretches to span the territory of the future myofiber (Fig. 1) (Rau et al., 2001). By the time that Eve declines at Stage 15, the DA1 muscle of wild-type embryos contains an average of 11 nuclei (Menon et al., 2005) (Fig. 1Q). As a control, we confirmed that the DA1 founder cell undergoes no fusion in mbc mutant embryos (Fig. 1D-F,P) (Beckett and Baylies, 2007; Menon et al., 2005; Schroter et al., 2004). By comparison, bi- and tri-nucleate muscle precursors are reproducibly observed in embryos lacking sns (Fig.

**Fig. 1. Fusion of the Eve-expressing DA1 founder cell in various mutant embryos.** (A-O) Abdominal segments 3-5 are shown in embryos stained with anti-Eve to mark the nuclei of the DA1 muscle and two pericardial cells per hemisegment. (A-C) Wild-type, (D-F) mbc^{D11.2}, (G-I) sns^{XB3}/sns^{XB3}, (J-L) Df(1)w67k30/Y and (M-O) lmd^{d1}/lmd^{d1}. The founder cell for DA1 remains mononucleate in embryos lacking mbc, kirre and rst [Df(1)w67k30], or lmd at developmental stages when significant fusion is observed in wild-type embryos (arrows). In embryo mutant for sns, by contrast, the Eve-expressing DA1 founder cell undergoes limited fusion to generate bi- or tri-nucleate syncitia (arrowheads). (P) The average number of DA1 nuclei per hemisegment was quantitated in late stage 15 embryos of each mutant genotype. (Q) The fusion profile of precursor formation in wild-type and sns^{XB3} embryos shown as the average number of DA1 nuclei per hemisegment. (R) The percentage of embryos observed with any hemisegments showing DA1 precursor formation. Scale bar: 20 μm.
However, embryos lacking both kirre and rst exhibit no fusion by late stage 15 (Fig. 1J-L,P) (Menon et al., 2005). Thus, precursor formation can occur through a Kirre/Rst-mediated process that is independent of Sns. This fusion is not the result of Kirre- or Rst-mediated homotypic interaction between founder cells, as it is not observed in embryos lacking lmd (Fig. 1M-O,P) (Menon et al., 2005) in which FCMs are not specified (Duan et al., 2001; Furlong et al., 2001; Ruiz-Gomez et al., 2002). Thus, the fusion events that occur in sns mutant embryos are dependent on the presence of FCMs.

Rst is expressed in both founder cells and FCMs (Galletta et al., 2004; Strunkelnberg et al., 2001) and is capable of interacting homotypically (Dworak et al., 2001; Galletta et al., 2004). To address whether it is sufficient to direct interaction between founder cells and FCMs in the absence of sns, the Eve-positive nuclei in the DA1 founder cell were quantitated in embryos mutant for sns alone and rsterC1; snsXB3, hbs2593 double mutants. At stages 15 and 16, embryos of both genotypes exhibited multiple Eve-positive nuclei (Fig. 2A,B), indicating that Rst does not substitute for Sns in the FCMs.

Like Sns (Bour et al., 2000) and Rst (Strunkelnberg et al., 2001), the IgSF protein Hibris is detected in all or a large subset of FCMs (Artero et al., 2001). Studies have reported that Hbs acts antagonistically to Sns (Artero et al., 2001). By contrast, an analysis of DA1 similar to that described above revealed a lower number of Eve-positive nuclei in sns, hbs double mutant embryos (Menon et al., 2005). To resolve this apparent difference, we examined the Eve-expressing DA1 founder cell, the Kr-expressing DA1 and dorsal oblique muscle 1 (DO1) founder cells and the Nau-expressing ventral acute muscle 1 (VA1) founder cell. Multiple DA1 nuclei were present in sns mutant embryos, as revealed by expression of Eve (Fig. 2C,E) and Kr (Fig. 2E). Multiple Kr-positive nuclei were also observed in DO1 (Fig. 2E). Finally multiple Nau-positive nuclei were apparent in VA1 (Fig. 2G). Thus, fusion occurs in the absence of sns in the DO1, DA1 and VA1 founder cells. By comparison, representative embryos revealed that DA1, DO1 and VA1 all remain mononucleate in most hemisegments of snsXB3, hbs2593 mutant embryos (Fig. 2D,F,H). We therefore conclude that Hbs substitutes for Sns in formation of the bi- and tri- nucleate precursors for muscles DA1, DO1 and VA1.

**Hbs has features and behaviors in common with Sns**

The above results indicate that Hbs functions redundantly with Sns in formation of muscle precursors. Consistent with this result, Hbs has several features in common with Sns. Hbs directs adhesion with S2 cells expressing Kirre protein at a rate comparable to that of Sns (Fig. 3A). Sns and Hbs also share similar modes of protein modification. In particular, Sns and Hbs are typical of many cell adhesion molecules in that their extracellular domains are modified by N-linked glycosylation (Fig. 3B). We previously demonstrated that Sns was phosphorylated on tyrosines when expressed pan-mesodernally in the embryo using mef2Gal4 and that these tyrosines impact the ability of Sns to drive myoblast fusion (Fig. 3C) (Kocherlakota et al., 2008). Analogous studies reveal that Hbs is similarly phosphorylated on tyrosines (Fig. 3C), and many of these tyrosines align with those in Sns (Fig. 3D). Lastly, the Sns and Hbs cytodomains both include two PxxP motifs, which have the potential to interact with SH2-domain-containing proteins and have been shown to play a role in Sns, one of which is closely aligned and highlighted (Fig. 3D) (Kocherlakota et al., 2008).
Hbs sequences can drive some myoblast fusion in Sns mutant embryos

Although endogenous Hbs acts positively to direct formation of bi and tri-nucleate muscle precursors, and has many features in common with Sns, it is unable to replace Sns in formation of mature myofibers. Possible explanations are that Hbs is spatially restricted, that it is present at lower levels and/or that it functions less efficiently than Sns. Alternatively, Hbs may lack sequences that mediate interactions crucial for progression beyond precursor formation, the ‘second step’ in myoblast fusion. To address these possibilities, we examined the ability of full-length Hbs and Hbs/Sns domain swaps to rescue fusion in sns mutant embryos when expressed either in the FCMs or pan-mesodermally under the control of snsGal4 (Kocherlakota et al., 2008) or mef2Gal4 (Fig. 4; see Fig. S3 in the supplementary material). These studies demonstrate that Hbs can act positively to direct myoblast fusion beyond the precursor stage, but inefficiently and only when overexpressed (Fig. 4D-F; see Fig. S3D-F,P in the supplementary material). Moreover, we infer from these results that the Hbs cytodomain must contain sequences capable of directing the necessary downstream events.

We next sought to determine why neither snsGal4- nor mef2Gal4-driven expression of UAS-hbs in a pattern that is spatially and temporally comparable to that of UAS-sns does not rescue myoblast fusion to completion. To evaluate the ability of Hbs extracellular, intracellular or transmembrane domains to impact fusion in sns mutant embryos, chimeric molecules were constructed in which corresponding domains of Sns and Hbs were swapped. Our rationale was that Hbs domains acting in a manner analogous to those of Sns should rescue to a comparable level. By contrast, Hbs sequences that act inefficiently should rescue less fusion and sequences that function specifically to antagonize Sns should not rescue at all. The sequences of all molecules were confirmed before injection, and are provided in Fig. S2 (see Fig. S2 in the supplementary material). UAS-SEHTC-HA includes the extracellular domain of Sns with the transmembrane and cytoplasmic domain of Hbs. UAS-SEHTC-HA includes the extracellular and transmembrane domains of Sns with the cytodomain of Hbs. Finally, UAS-HESTC-HA includes the Hbs ectodomain with the Sns transmembrane and cytodomains. We confirmed that protein levels from all transgenes were present at approximately equal levels, and that the domain swap constructs had no significant impact when overexpressed in wild-type embryos compared to that observed with UAS-sns-HA itself (data not shown). The extent of rescue for domain swap molecules was compared to that directed by UAS-hbs-HA (Fig. 4D-F) or UAS-sns-HA (Fig. 4P-R). Clearly, the Hbs ectodomain is capable of rescuing significant myoblast fusion in the context of the Sns transmembrane and cytodomains (Fig. 4M-O). Some unfused myoblasts were present and muscles were occasionally missing. However, we did not observe a dramatic impact of swapping these domains. The Sns extracellular and transmembrane domain in combination with the Hbs cytodomain, by comparison, rescued much less fusion than that observed with UAS-sns-HA or UAS-HESTC-HA, but more than that observed with UAS-hbs-HA alone (Fig. 4J-L compared with Fig. 4D-F). Similar results were observed with transgenes that swap the Hbs ectodomain with that of Sns in the context of the Hbs transmembrane and cytodomains. Most importantly, the latter two proteins are capable of directing fusion well beyond the precursor stage, and beyond that seen with full-length Hbs. Mef2Gal4, which drives expression pan-mesodermally and at a higher level than snsGal4, yielded similar results (see Fig. S3 in the supplementary material).

We did not observe significant or consistent pattern defects in these rescued embryos to suggest that the presence of Hbs or a balance between Sns and Hbs favors the formation of some muscles to the detriment of others. Rather, we observed a fairly consistent gradient in which fusion in all muscles was improved by swapping Sns sequences for those of Hbs. Furthermore, significant muscle formation was observed at stage 14 using UAS-hbs, compared with the sns loss-of-function mutation alone, suggesting that higher expression is a significant driving factor behind increased muscle formation in these rescued embryos (Fig. 4S-U). We conclude from these results that several domains within Hbs function inefficiently in directing myoblast fusion compared with the analogous sequences in Sns, but that these domains are nonetheless capable of driving significant fusion. We hypothesize that weak protein-protein
interactions between these Hbs domains and their downstream effectors limit the ability of endogenous Hbs to function, but that these interactions occur under Gal4-driven conditions in which higher levels of Hbs are present.

Hbs interacts genetically and physically with Sns

Published studies have shown that myoblast fusion defects in hbs mutant embryos decrease with the loss of one copy of sns (Artero et al., 2001), contributing to the conclusion that Hbs functions to limit the action of Sns, and implying that excess Sns is deleterious in some way. To first determine the consequences of misregulated or excess Sns, we overexpressed a functional UAS-sns transgene in the musculature of wild-type embryos using mef2Gal4. An sns-promoter reporter transgene (snslacZ) was incorporated to facilitate quantitation of unfused myoblasts (Kocherlakota et al., 2008). As shown in Fig. 5A, excess Sns has little impact on muscle pattern or on the number of unfused myoblasts compared to controls. We then compared the number of unfused myoblasts in hbs2593/hbs459 trans-heterozygous embryos, which are hypomorphic and null alleles of hbs, respectively (Artero et al., 2001), and hbs2593/hbs459 mutant embryos that were heterozygous for sns257 (Fig. 5B). This analysis used an sns257; hbs2593 double mutant that has been described (Artero et al., 2001; Menon et al., 2005). To eliminate a potential contribution to this mutant phenotype by mutations elsewhere on the sns257; hbs2593 recombinant chromosome, we also generated sns257, hbs459 (see Fig. S1 in the supplementary material). Unfused myoblasts were preferentially detected by staining for Sns and quantitated. In short, the limited number of unfused myoblasts in mutant embryos for sns257 actually increased rather than decreased upon removal of one copy of sns. Thus the loss of hbs is deleterious to myoblast fusion and removal of one copy of sns enhances this effect, supporting our model that sns and hbs both act positively to drive myoblast fusion.

Mechanistically, Hbs can substitute for Sns in early myoblast fusion and rescue extensive myoblast fusion when overexpressed in sns mutant embryos, yet it interferes with fusion when overexpressed in whole or in part in a wild-type embryo (Artero et al., 2001; Dworak et al., 2001). One explanation for this behavior was provided by proteomic studies that revealed an interaction between Sns and Hbs in the somatic musculature (data not shown). To validate this potential interaction, S2 cells were transiently transfected with combinations of pUAST-hbs, pUAST-sns-Flag and pWAGal4, the interacting proteins identified by immunoprecipitation and immunoblot (Fig. 5C). Hbs co-precipitated with Sns in extracts from cells expressing Sns and Hbs but not from cells expressing Hbs alone. This interaction occurs in cis, as Hbs was not detected by interaction with Sns upon mixing of independently transfected cells before lysis preparation (data not shown). As observed for the interaction between the IgSF proteins CAM-related/downregulated by oncogenes (Cdo) and Brother of Cdo (Boc) (Kang et al., 2002), both the extracellular and intracellular domains of Sns were capable of mediating interaction.
with Hbs (see Fig. S4 in the supplementary material). To confirm this association in the embryonic mesoderm, recombinant flies containing both UAS-hbs and UAS-sns-V5 transgenes were mated to flies expressing mef2Gal4. Lysates were prepared from Stage 9-15 embryos and analyzed by immunoprecipitation. Again, Hbs efficiently co-precipitated with Sns in lysates from embryos expressing Hbs and Sns-V5 but not from embryos expressing only Hbs (Fig. 5C). Thus, Sns and Hbs are present in a hetero-oligomer in S2 cells and in the developing embryonic musculature. Similar studies using V5 and HA-tagged Sns (Materials and methods) revealed that Sns was also present in homo-oligomers in S2 cells and in embryos (Fig. 5D). In combination with the observation that Hbs functions less efficiently than Sns in driving myoblast fusion (Fig. 4), this finding supports a scenario in which excess Hbs could sequester Sns in a less functional or nonfunctional complex, leading to the observed defects in myoblast fusion upon Hbs overexpression (Artero et al., 2001; Dworak et al., 2001).

**Fusion to form muscle precursors requires the Sns or Hbs cytodomain**

One remaining issue in understanding the abilities of Hbs and Sns to direct precursor formation is whether intracellular events are necessary for this early event. To address this question, we employed an sns transgene that mimics the molecular lesion present in the EMS-induced sns^{rost20-5} allele (Paululat et al., 1995), which lacks a functional cytoplasmic domain and is unable to drive myoblast fusion...
in sns mutant embryos (Kocherlakota et al., 2008), and a hbs transgene in which the cytoplasmic domain is truncated in a region homologous to that of the sns20-5 lesion (UAS-hbsΔICD-HA). These transgenes under control of mef2Gal4 were then assayed for their ability to rescue formation of Eve-positive (DA1) or Kr-positive (DO1) bi- and tri-nucleate muscle precursors in sns20-5, hbs2593 double mutant embryos. Even by stage 15, past the time at which multinucleate precursors are first seen in sns mutant embryos (Fig. 1; Fig. 6E), no fusion was observed upon expression of either transgene (Fig. 6A,C,F). In fact, we were unable to detect fusion in the DA1 or DO1 muscles as late as mid-stage 16 upon expression of truncated Sns or Hbs (Fig. 6B,D), after which the reporters were no longer detectable. We interpret these data to indicate that the first step in myoblast fusion, in which a muscle precursor is formed from fusion between a founder cell and one or two FCMs, requires interactions that occur downstream of Sns and Hbs and, in conjunction with the data of Fig. 3, are mediated by their cytoplasmic domains.

**DISCUSSION**

These results address an important issue in our understanding of myoblast fusion and the relative contributions of Sns and Hbs, clarifying why precursor formation is dependent on the Sns receptors Kirre and Rst but independent of Sns. Precursors clearly form in sns mutants and rst; sns double mutants, but do not form in sns, hbs double mutants. Possible redundancy between sns and hbs has been reported (Menon et al., 2005), but these studies addressed hbs function in only one aspect of muscle development and did not resolve conflicts with the previously reported function of hbs. Our finding that Hbs is inefficient in directing fusion beyond precursor formation may account for the previously observed consequences of UAS-hbs overexpression (Artero et al., 2001). Moreover, genetic interactions between hbs and sns observed at later stages of muscle development clearly indicate that Hbs acts positively to direct myoblast fusion in a manner similar to that of Sns. Finally, the cytodomain of either Hbs or Sns must be present for precursor formation, suggesting that intracellular events downstream of these cell surface proteins are important during or before fusion for generation of muscle precursors.

**A revised role for Hbs**

Sns and Hbs function redundantly in the initial fusion event between founder cells and FCMs. As observed in other mutants (Beckett and Baylies, 2007), precursor formation in sns mutant embryos is delayed over that occurring in wild-type embryos, but is readily observed in stage 13 embryos in at least some segments. By contrast, no fusion was observed by late stage 15 in sns, hbs double mutant embryos. Although we cannot eliminate the possibility of a temporal delay of fusion in sns, hbs double mutants because reporter expression declines after this stage, we favor a model in which a crucial first step is not occurring in the absence of both Sns and Hbs. Using new FCM reporters that facilitate quantitation of unfused myoblasts, re-examination of the hbs loss-of-function phenotype reveals that the loss of one copy of sns actually worsens the hbs mutant phenotype, as expected if these proteins have some functional redundancy. Finally, both snsGal4 and mef2Gal4 directed Hbs can drive a significant amount of fusion in sns mutants, arguing that Hbs is capable of directing fusion beyond precursor formation.

Although Hbs can rescue the sns mutant phenotype beyond precursor formation, replacing any domain of Hbs with the comparable domain of Sns improves the ability of the chimeric protein to rescue fusion over that achieved by Hbs alone. The activity of the Hbs cytodomain is most dramatically different from that of Sns, providing an explanation for the observation that intact Hbs or a membrane-anchored Hbs cytoplasmic domain both interfere with myoblast fusion in wild-type embryos (Artero et al., 2001; Dworak et al., 2001). Rather than acting as an antagonist of Sns, these high levels of Hbs probably interfere competitively with endogenous Sns. First, an excess of Hbs may drive its interaction with a limiting component that is normally used more efficiently by Sns. Alternatively, given their ability to form hetero- and homodimers in vivo, excess Hbs may sequester Sns in a less functional form. Although our data do not fully resolve this issue, the co-localization of Hbs and Sns is consistent with the latter model (Artero et al., 2001). Of note, dimer formation between the related IgSF proteins Boc and Cdo can be directed by sequences in both the extracellular and intracellular domains (Kang et al., 2002), and both the extracellular and intracellular domains of Sns are capable of mediating its interaction with Hbs, raising the possibility that either full-length Hbs or a membrane-anchored cytodomain may sequester Sns under conditions of overexpression.

**Implications for the regulation of Sns**

The finding that Hbs functions positively but much less efficiently than Sns in directing later rounds of myoblast fusion provides an explanation for the previously observed behavior of Hbs in
overexpression assays (Artero et al., 2001; Dworak et al., 2001). Additionally, our data appear to be inconsistent with a model in which excess Sns is deleterious, as inferred if a decrease in sns copy number compensates for the loss of hbs (Artero et al., 2001). We cannot eliminate the possibility that Sns activity is negatively regulated. Possible mechanisms could include limitations in the machinery for tyrosine phosphorylation, such that unphosphorylated Sns even in excessive amounts would be unable to transduce a signal to downstream events. Downstream targets of Sns may also be limiting, such that no further activation of the pathway can be accomplished by Sns. We also note that Sns protein is transient, appearing just before fusion and being eliminated shortly thereafter. Despite the issue of whether Sns activity is regulated in some fashion, our data are not consistent with a model in which its activity is negatively regulated by endogenous Hbs.

**Hbs does not replace Sns: implications for signal transduction mediated by IgSF proteins**

Current models for myoblast fusion suggest that it occurs in two steps that differ genetically and/or temporally. Consistent with the two genetically distinct steps, fusion does not occur in embryos mutant for genes encoding the guanine nucleotide exchange factors Schizo (Chen et al., 2003), Mbc (Beckett and Baylies, 2007; Menon et al., 2005; Schroter et al., 2004) or, as discussed herein, Duf and Rst (Menon et al., 2005). By contrast, precursor formation is observed in embryos lacking the Hem-2/Nap1 homolog Kette (Schroter et al., 2004), the Kirre-associated protein Rols (Chen and Olson, 2001; Rau et al., 2001), the Arp14D/66B regulators WASp and Vrp1 (Berger et al., 2008; Kim et al., 2007; Massarwa et al., 2007; Schafer et al., 2007) or, as described herein, Sns. These data support a model in which the molecular requirements for precursor formation differ from those for subsequent myotube formation (Berger et al., 2008; Doberstein et al., 1997; Kesper et al., 2007). An alternative model, using three dimensional analyses and quantitating fusing myoblasts over time, revealed that fusion occurs in two temporal phases, comprising an initial phase of limited fusion between cells that are in close proximity and a second phase when most myoblast fusion occurs (Beckett et al., 2007). Moreover, precursor formation is temporally delayed in embryos lacking molecules such as Rols and Kette, suggesting that these molecules do influence the first step in fusion (Beckett et al., 2007; Richardson et al., 2008).

The present study does not address whether the genetic requirements for precursor formation differ from those for subsequent rounds of fusion, or whether these steps utilize the same set of proteins. Our data do not eliminate the possibility of two distinct genetic steps, with Sns and Hbs acting redundantly in precursor formation but not in later events. Hbs is capable of directing precursor formation in the absence of Sns. However, the ability of Hbs to drive fusion beyond precursor formation when in excess, and the observation that removal of one copy of sns enhances fusion defects in hbs mutants, suggests that Hbs can assist in later rounds of myoblast fusion. These data are consistent with models in which molecular interactions in precursor formation and subsequent fusion differ kinetically but not genetically (Beckett et al., 2007; Richardson et al., 2008). One possibility, independent of the process of fusion itself, is that Sns and Hbs differ in their ability to drive FCM cell migration. Although the role of cell migratory behavior in myoblast fusion is unclear, the ability to migrate may contribute to the rate of fusion (Beckett et al., 2007; Richardson et al., 2008). While these questions remain to be addressed, the present study advances our understanding of fusion by resolving the interaction of two proteins that function early in the process, thereby providing additional perspectives for sorting out the different mechanisms of myoblast fusion.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/7/1159/DC1

**References**


