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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, consistent with its ability to drive myoblast fusion, 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 *neph1-4* 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 Arf51F (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 are essential for myoblast fusion (Geisbrecht et al., 2008; Hakeda-Suzuki et al., 2002). Activated Rac1 then modulates polymerization of Actin 5C (F-actin) through the Arp14D/66B (Arp2/3) complex via SCAR (Berger et al., 2008; Richardson et al., 2007) and the regulatory factor Hem (Kette) (Hummel et al., 2000; Schroter et al., 2004). Arp14D/66B-directed actin polymerization is also regulated in the FCMs through the action of WASp and the FCM-specific WASp-interacting protein Verprolin 1 (Vrp1; also termed *D-wip* and *solitary*, or *sltr*) (Berger et al., 2008; Kim et al., 2007; Massarwa et al., 2007; Schafer et al., 2007). Consistent with the involvement of actin remodeling proteins, dynamic foci of Actin 5C are present at sites of myoblast fusion and modulated by these proteins (Kesper et al., 2007; Richardson et al., 2007). Although Kim et al. (Kim et al., 2007) suggest that the WASp/Vrp1 complex is connected to the Sns cytodomain via the SH2-SH3 adaptor protein Crk (Galletta et al., 1999), the biochemical interactions necessary for activation of the pathway are not well understood. Indeed, multiple redundant functional domains in the Sns cytodomain have the potential to mediate a spectrum of interactions (Kocherlakota et al., 2008).

To better understand the role of Sns in myoblast fusion, identify mechanisms through which fusion can occur in its absence, and resolve the relative contribution of Hbs, we undertook a detailed examination of Sns and Hbs. We report herein that Hbs acts positively to direct myoblast fusion, is capable of driving significant fusion even in the absence of Sns and functions interchangeably with Sns in the first fusion events between founder cells and FCMs. Re-examination of the genetic interaction between *sns* and *hbs* with new methods of visualization and quantitation also support a model in which Hbs functions positively to direct myoblast fusion. The ability of chimeric proteins of Sns and Hbs to rescue fusion in *sns* mutant embryos supports a model in which Hbs functions less efficiently than Sns, a limitation that rests primarily within its cytoplasmic domain. Finally, our data establish conclusively that all fusion requires signaling pathways that are downstream of Sns and Hbs.

MATERIALS AND METHODS

Fly stocks

The following mutant alleles have been reported: sns^{XB3}, $sns^{Zf1.4}$, *Df(2R)BB1* (Bour et al., 2000), $mbc^{D11.2}$ (Erickson et al., 1997), lmd^{1} (Duan et al., 2001), *Df(1)w67k30* (Ruiz-Gomez et al., 2000), rst^{irreC1} (Strunkelnberg et al., 2001), hbs^{459} and hbs^{2593} (Artero et al., 2001). Stocks described elsewhere include *mef2Gal4* (Ranganayakulu et al., 1998), UAS-*sns-HA*, *snslacZ* and *snsGal4* (Kocherlakota et al., 2008) and UAS-*hbs* (Artero et al., 2001). The sns^{s660} allele was a gift from Elizabeth Chen. The sns^{D1}, hbs^{459} double mutant stock was generated by EMS mutagenesis of an isogenized hbs^{459} chromosome to generate mutations in *sns* (see Fig. S1 in the supplementary material). The molecular lesions in sns^{s660} and sns^{D1} 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 sns^{XB3} or $sns^{Zf1.4}$ and balanced with *CyO, P{ry[+t7.2]=en1}*wg^{en11} 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 *Eco*RV-*Sal*I fragment that includes the entire *kirre-HA* coding sequence from pRmHa3-*kirre-HA* (Galletta et al., 2004) was sub-cloned into pBSK, recovered as a *Not*I-*Kpn*I fragment and cloned into pUAST (Brand and Perrimon, 1993). To generate pUAST-*hbsΔICD-HA* an HA epitope tag followed by a stop codon and an *Xba*I restriction site were introduced after AA 1114 of Hbs. The pUAST-*sns-V5*, pUAST-*sns-Flag*, pUAST-*hbs-HA*, pRmHa3-*sns-V5*, pRmHa3-*hbs-HA* and pRmHa3-*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′-CAGCGCCGCAA-GAAAGTGTCTCAGAGCGAAGCGGA-3′) for pUAST-*SETHC-HA*, (5′-CATGGCTGCTGAGGTGATTCCAATTATGACCAAATTCGGTGT-GTTGC-3′) for pUAST-*SEHTC-HA* and (5′-GAATGCCGCCAGAGA-GATGCCAATGATCATCACATTGGGCAGTTCGTC-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 *Afl*II and *Xba*I. The chimeric sequence for *HESTC-HA* was substituted for Hbs in pUAST-*hbs* (Artero et al., 2001) using *Bgl*II and *Xba*I. An HA epitope tag, stop codon and *Xba*I 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 of *hbs* 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. Kiehart), Even skipped (Eve, 1:1000, M. Frasch), Pericardin (Prc, 1;10, Developmental Society Hybridoma Bank), Kruppel (Kr, 1:300, East Asian Distribution Center for Segmentation Antibodies at National 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 (Eve and Kr) or four (Nau) abdominal hemisegments were analyzed per embryo. For rescue of sns^{XB3}, hbs^{2593} double mutants, six (Eve 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 Biomedicals) 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 chimeras 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.

DEVELOPMENT

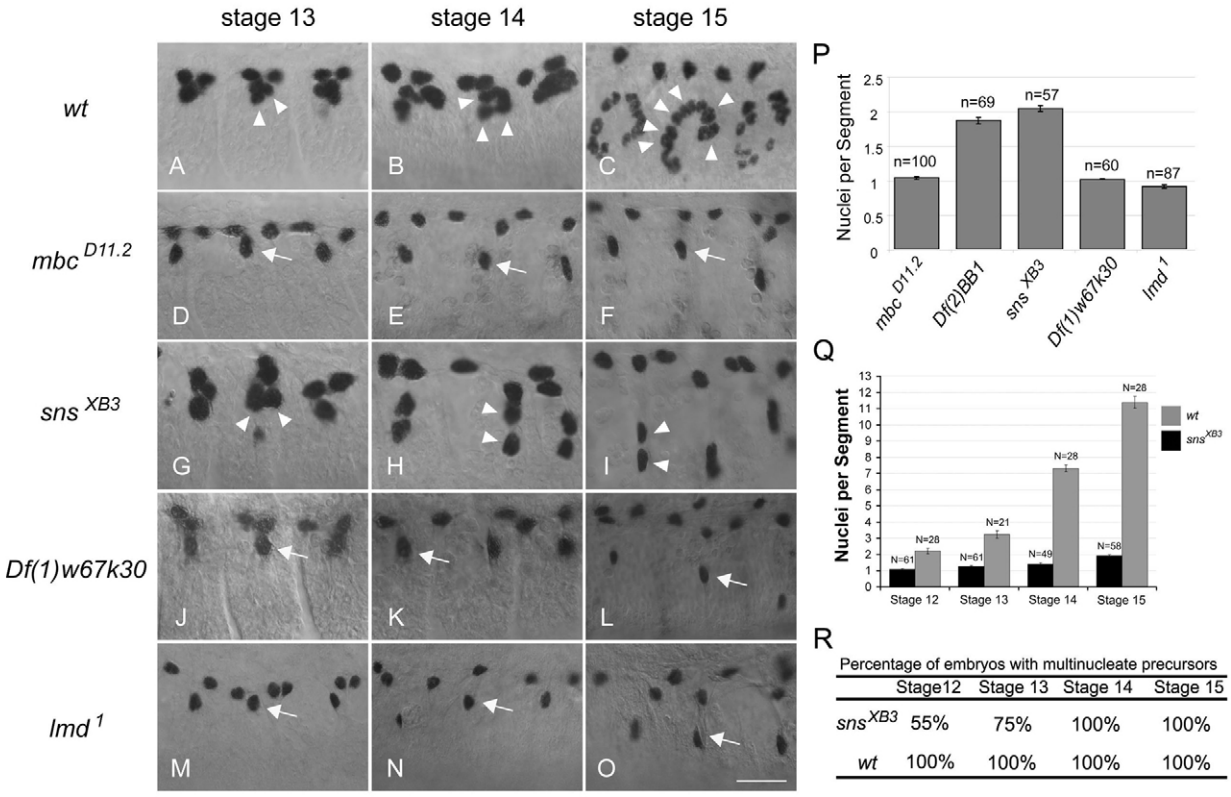

Fig. 1. Fusion of the Eve-expressing DA1 founder cell in various mutant embryos. (**A-O**) Abdominal segments 3-5 are shown in embryos stained with anti-Eve to mark the nuclei of the DA1 muscle and two pericardial cells per hemisegment. (A-C) Wild-type, (D-F) $mbc^{D11.2}/mbc^{D11.2}$, (G-I) sns^{XB3}/sns^{XB3}, (J-L) *Df(1)w67k30/Y* and (M-O) lmd^{1}/lmd^{1}. 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 embryos 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.

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-s*ns-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 $CuSO_4$. 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.

Immunoprecipitations, PNGaseF digestion and immunoblotting

Immunoprecipitations from embryos or transfected S2 cells were performed as described (Balagopalan et al., 2006) using anti-V5 resin (Invitrogen, Carlsbad, CA), anti-HA clone3F10-affinity matrix (Roche Applied Science, Indianapolis, IN) or anti-FLAG-M1-agarose (Sigma, St Louis, MO), as indicated. Proteins were eluted either by boiling in Laemmli buffer, for immunoblots, or by incubation with an HA peptide for PNGaseF digestion. PNGaseF (*Elizabethkingia meningosepticum*, EMD chemicals, Gibbstown, NJ) digestion was as described (Hanford et al., 2004). Immunoblots used rabbit anti-Hbs (1:1000; gift of Mary Baylies), mouse anti-phosphotyrosine (1:1000; Upstate Biotechnology), horseradish-peroxidase-conjugated, anti-mouse, anti-rabbit or anti-rat (1:5000; GE Healthcare Lifesciences), anti-HA (1:3000; Roche, Indianapolis, IN), anti-V5 (1:5000; Invitrogen, Carlsbad, CA), anti-HA HRP conjugate (1:5000; Roche, Indianapolis, IN) anti-V5 HRP conjugate (1:5000; Invitrogen, Carlsbad, CA), and anti-FLAG (1:5000; Sigma, St Louis, MO).

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.

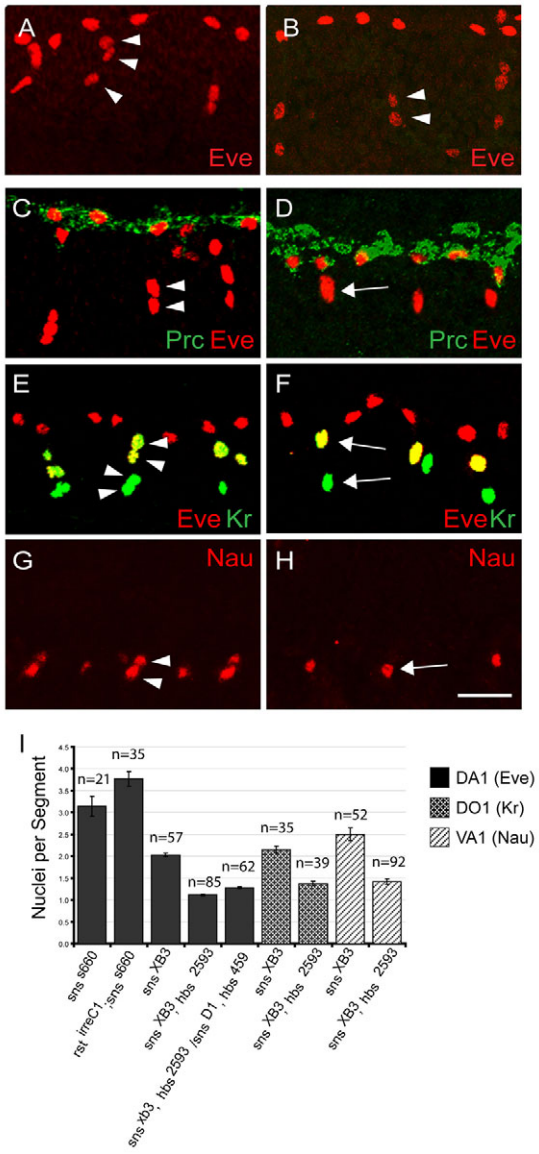

Fig. 2. Hbs, but not Rst, substitutes for Sns in directing precursor formation. (**A-H**) Three representative abdominal segments of late stage 15 embryos. (A-F) Eve marks DA1 and pericardial cell nuclei. A similar amount of fusion is observed in *sns*^{s660} mutants (A) and *rst*^{irreC1}; *sns*^{s660} double mutants (B). (C,D) Pericardin (Prc) staining identifies the Eve-expressing pericardial cells. Multinucleate DA1 precursors are present in *sns*^{XB3} mutants (C, arrowheads) but not in *sns*^{XB3}, *hbs*^{2593} recombinants (D, arrows). (E,F) Eve and Kr expression identify nuclei in DA1, whereas Kr expression identifies nuclei in DO1. Fusion to form the DA1 and DO1 precursor is observed in *sns*^{XB3} mutant embryos (E, arrowheads) but not *sns*^{XB3}, *hbs*^{459} recombinants (F, arrows). (G,H) Nau expression marks nuclei of the VA1 muscle. Again, fusion is observed in *sns*^{XB3} mutants (G, arrowheads) but not in *sns*^{XB3}, *hbs*^{2593} recombinants (H, arrow). (**I**) The average number of nuclei per hemisegment is shown for DA1 (Eve), DO1 (Kr) and VA1 (Nau) in the indicated genotypes. Scale bar: 20 μm.

1G-I,P). Although delayed compared with the initial fusion event observed in wild-type embryos, this Sns-independent fusion was observed as early as stage 12. It was observed consistently across abdominal hemisegments by stage 15 (Fig. 1Q-R). Like *mbc*, 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*^{s660} alone and *rst*^{irreC1}; *sns*^{s660} 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 *sns*^{XB3}, *hbs*^{2593} 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-mesodermally 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).

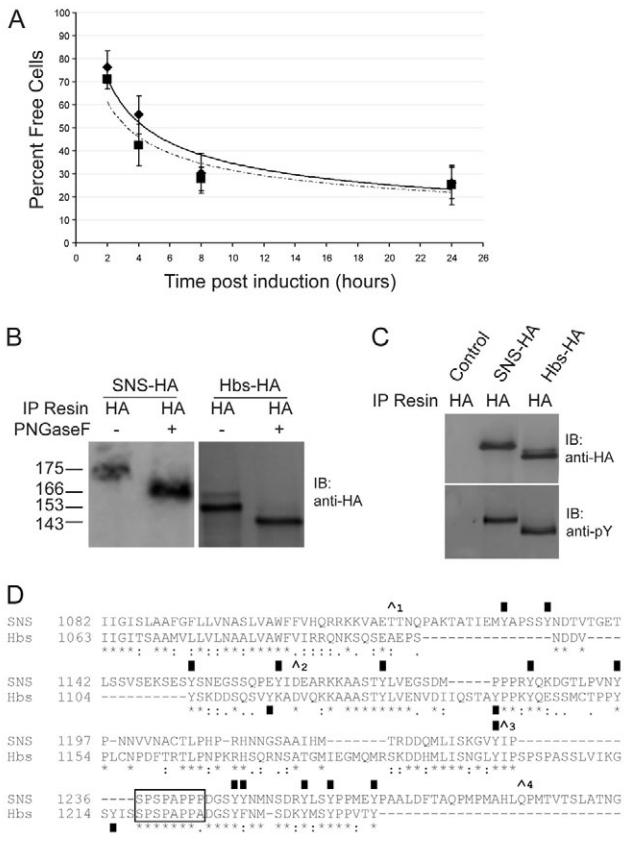

Fig. 3. Hbs and Sns have comparable aggregation behavior with Kirre-expressing cells and undergo similar biochemical modifications. (**A**) Kinetics of trans-heterotypic aggregation of S2 cells expressing Kirre co-cultured with S2 cells expressing Sns (diamonds, bold line) and S2 cells expressing Hbs (squares, dashed line). (**B**,**C**) Sns-HA or Hbs-HA expressed pan-mesodermally with *mef2Gal4* and immunoprecipitated with anti-HA resin. (B) Immunoblots of untreated (Mock) or PNGaseF-treated Sns-HA and Hbs-HA reveals modification by N-linked glycans. (C) Immunoblots of Sns-HA, Hbs-HA or control (*mef2Gal4*/+) samples probed with anti-HA or anti-phosphotyrosine antibodies. (**D**) Alignment of transmembrane and cytodomain sequences from Sns and Hbs. The entire Hbs cytodomain is shown. Only the membrane proximal half of the Sns cytodomain is shown. The caret positions correspond to (1) AA1113, (2) AA1164, (3) AA1232 and (4) AA1278 of SNS, and served as deletion endpoints in a structure/function analysis of Sns. Conserved sequences from Hbs, important PxxP motifs and tyrosine residues crucial for Sns function are highlighted.

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-*SETHC-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

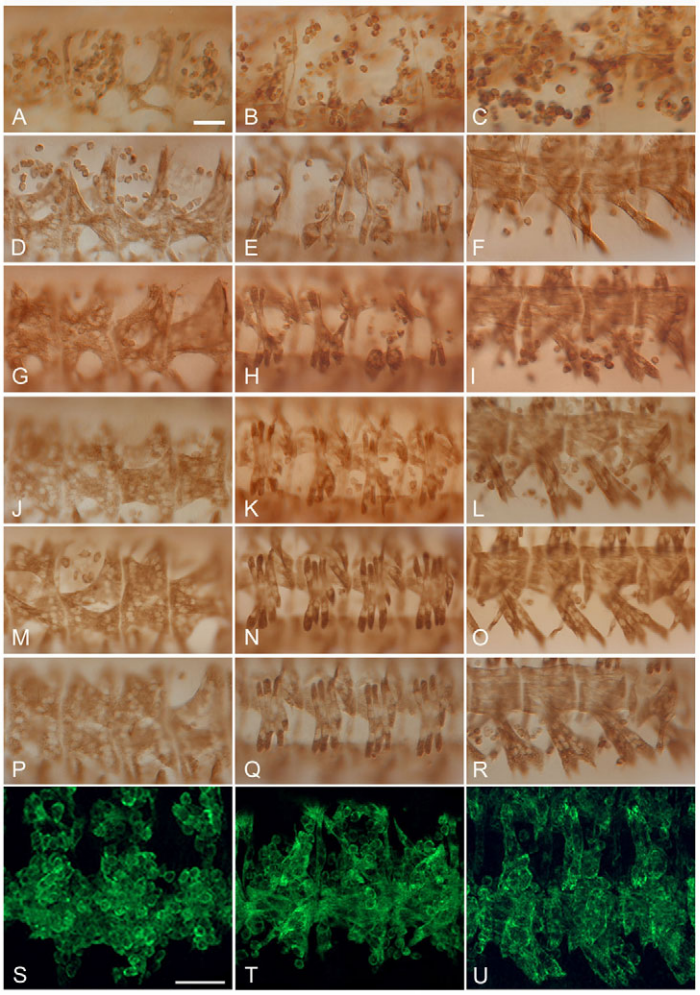

Fig. 4. Comparison of the ability of various Sns-Hbs chimeric proteins to rescue the *sns* mutant myoblast fusion defect. (**A-U**) Stage 16 embryos stained with anti-myosin heavy chain antibody. In all orientations, anterior is to the left and dorsal is up. (A,D,G,J,M,P) Dorsolateral view; (B,E,H,K,N,Q) lateral view; (C,F,I,L,O,R) ventrolateral view. Expression of the indicated transgenes (for sequences, see Fig. S2 in the supplementary material) was directed in $sns^{Zf1.4}/sns^{Zf1.4}$ mutant embryos with *snsGal4* at 25°C. (S-U) Ventrolateral views of sns^{XB3} mutant (S), and rescued $sns^{zf1.4}$ mutant embryos using *snsGal4* to drive expression of UAS-*hbs* (T) or UAS-*sns* (U). Scale bar: 20 μm.

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 hbs^{2593}/hbs^{459} trans-heterozygous embryos, which are hypomorphic and null alleles of *hbs*, respectively (Artero et al., 2001), and hbs^{2593}/hbs^{459} mutant embryos that were heterozygous for sns^{XB3} (Fig. 5B). This analysis used an sns^{XB3}, hbs^{2593} 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 sns^{XB3}, hbs^{2593} recombinant chromosome, we also generated sns^{D1}, hbs^{459} (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 embryos mutant for *hbs* 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 lysate 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

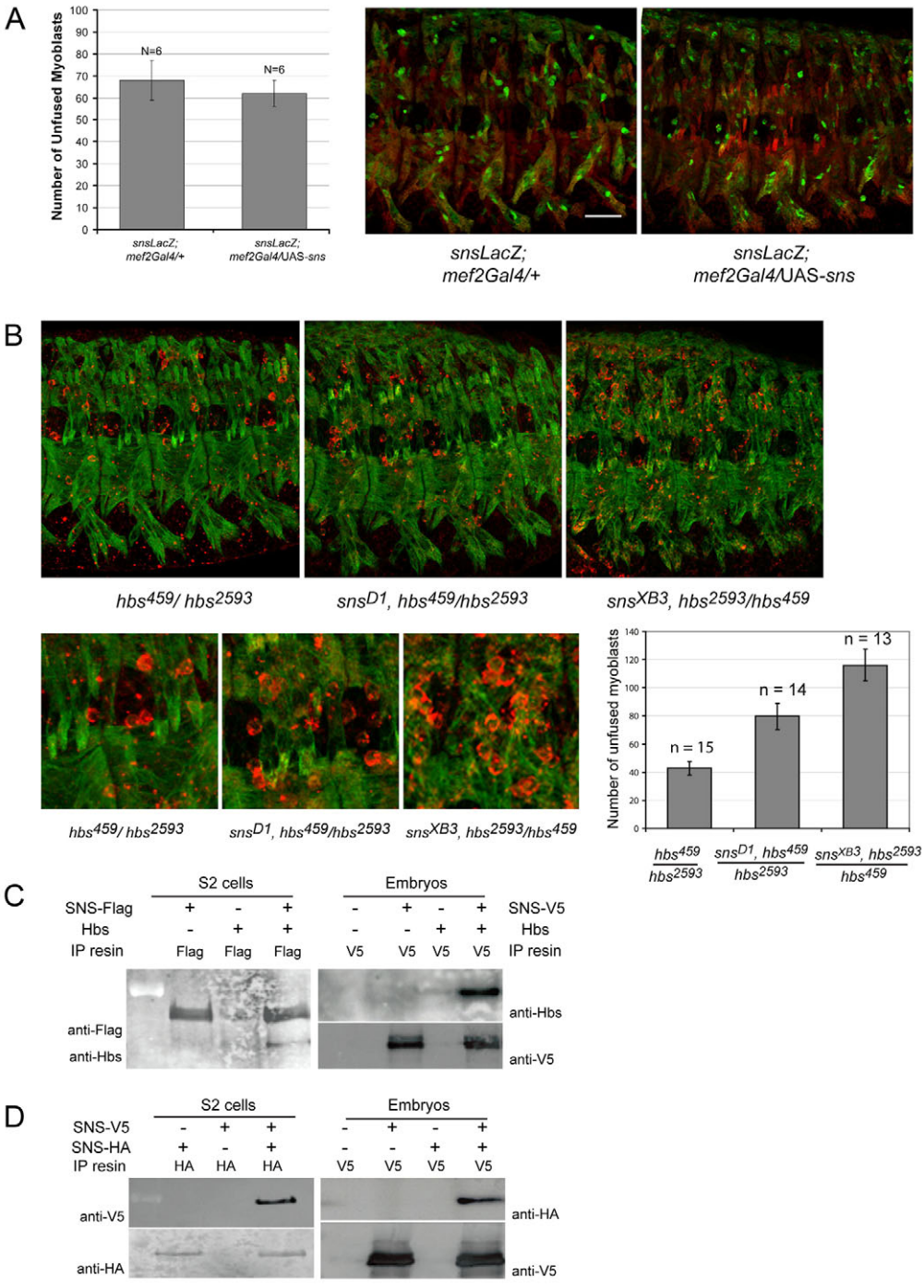

Fig. 5. Sns interacts with Hbs genetically and biochemically. (**A**) The average number of unfused myoblasts was determined by staining for β-galactosidase and quantitated in four contiguous abdominal segments of six embryos for control (*snslacZ/+; mef2Gal4/+*) and test (*snslacZ/+; mef2Gal4*/UAS-*sns*). Representative late stage 15 embryos fluorescently stained for myosin heavy chain (red) and β-galactosidase (green) are shown to the right. (**B**) Late stage 15 embryos of the indicated genotypes immunofluorescently stained for Sns (red) to highlight unfused myoblasts, and myosin heavy chain (green) to mark muscles. The bar chart shows the average number of unfused myoblasts for the indicated genotypes, quantitated in four contiguous abdominal segments per embryo. (**C**) In the left-hand panel, expression of pUAST-*sns-Flag* and/or pUAST-*hbs* was under the control of *actinGal4* (pWAGal4) in transiently transfected S2 cells. In the right-hand panel, expression of UAS-*sns-V5* and/or UAS-*hbs* was directed pan-mesodermally in embryos using *mef2Gal4*. Sns was immunoprecipitated from lysates using the indicated epitope tag, and the immunoblots probed with anti-flag, anti-V5 or anti-Hbs as indicated. (**D**) In the left-hand panel, S2 cells were transiently transfected with pUAST-*sns-V5* and/or pUAST-*sns-HA*. Expression was directed by *actin*Gal4 (pWAGal4). In the right-hand panel, expression of UAS-*sns-V5* and/or UAS-*sns-HA* was directed pan-mesodermally in embryos using *mef2Gal4*. Sns was immunoprecipitated from lysates using the indicated epitope tag, and the resulting immunoblots probed with anti-HA or anti-V5 as indicated. Scale bar: 20 μm.

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 $sns^{rost20\text{-}5}$ 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 $sns^{20\text{-}5}$ 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 sns^{XB3}, hbs^{2593} 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.

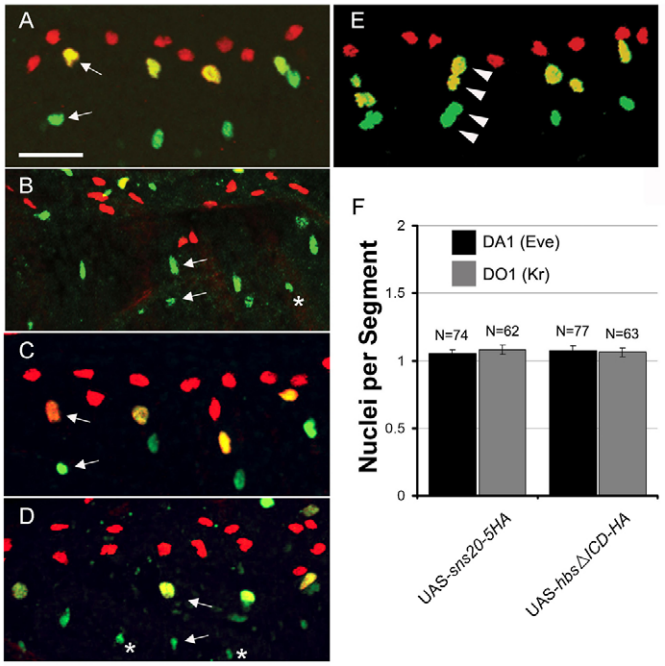

Fig. 6. The Sns extracellular domain alone is not able to direct precursor formation. (**A-E**) Embryos were immunofluorescently stained with anti-Eve and anti-Kr antibodies. (A-D) Expression of the UAS-*sns20-5HA* transgene (A,B) or the UAS-*hbsΔICD-HA* transgene (C,D) were driven pan-mesodermally with the *mef2Gal4* driver in an sns^{XB3}, hbs^{2593} mutant background. Note the presence of single Eve-positive or Kr-positive nuclei per hemisegment (arrows). The asterisks in B and D indicate the presence of anti-Kr staining not associated with the mesoderm. (E) Mid-stage 15 sns^{XB3} mutant embryo; note the regular occurrence of multiple Eve-positive or Kr-positive nuclei per hemisegment (arrowheads). (**F**) The average number of nuclei per hemisegment for abdominal segments 2-7 was calculated for the DA1 muscle (Eve-positive, Kr-positive) and the DO1 (Kr-positive) muscle for embryos where SNS20-5HA or HbsΔICD-HA expression was directed in sns^{XB3}, hbs^{2593} mutants. Scale bar: 20 μm.

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 *sns*Gal4 and *mef2*Gal4 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

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