

Bidirectional Notch activation represses fusion competence in swarming adult *Drosophila* myoblasts

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SUMMARY

A major aspect of indirect flight muscle formation during adult *Drosophila* myogenesis involves transition of a semi-differentiated and proliferating pool of myoblasts to a mature myoblast population, capable of fusing with nascent myotubes and generating mature muscle fibers. Here we examine the molecular genetic programs underlying these two phases of myoblast differentiation. We show that the cell adhesion proteins Dumbfounded (Duf) and Sticks and stones (Sns), together with their paralogs Roughest (Rst) and Hibris (Hbs), respectively, are required for adhesion of migrating myoblasts to myotubes and initiation of myoblast-myotube fusion. As myoblasts approach their myotube targets, they are maintained in a semi-differentiated state by continuous Notch activation, where each myoblast provides the ligand Delta to its neighbors. This unique form of bidirectional Notch activation is achieved by finely tuning the levels of the ligand and receptor. Activation of Notch signaling in myoblasts represses expression of key fusion elements such as Sns. Only upon reaching the vicinity of the myotubes does Notch signaling decay, leading to terminal differentiation of the myoblasts. The ensuing induction of proteins required for fusion enables myoblasts to fuse with the myotubes and give rise to subsequent muscle fiber growth.

KEY WORDS: Notch, Myoblast fusion, Myogenesis, *Drosophila*

INTRODUCTION

Multinucleated muscle cells are characteristic of the musculature of most multicellular organisms, accommodating the need to generate large and continuous fibers. Generation of myofibers from individual myoblasts has been studied in great detail in the *Drosophila* embryo, in which analysis of mutants has allowed the identification of key players. This is a complex process, involving the specification of cells that will serve as founders for each of the muscles, definition of myoblasts, and the attraction of myoblasts toward founder cells. Once the two cell types are in close contact, cell fusion is executed via an elaborate machinery involving cell adhesion, formation of fusion pores between the attached cells and dissolution of cell membranes (Haralalka and Abmayr, 2010). Which aspects of this process are unique to the *Drosophila* embryo, and what mechanisms will turn out to be common to all muscle fusion processes? One may expect the specification of distinct muscle cells to be more divergent for different organisms, whereas the actual machinery of fusion is likely to be more universal in nature (Moore et al., 2007; Laurin et al., 2008; Rochlin et al., 2010).

The somatic musculature of *Drosophila* embryos is formed from two subpopulations of myogenic mesoderm myoblasts, founder cells and the more numerous fusion-competent myoblasts (FCMs). Each of the 30 or so muscles of an embryonic hemisegment arises from a single, specific founder cell that expresses a distinct combination of transcription factors. Sequential rounds of fusion of each founder cell with neighboring FCMs generates myofibers of characteristic shape and size, in which all nuclei assume the specific muscle fate of the original founder cell. The overall number of myoblasts that are initially defined within the myogenic

mesoderm is sufficient to provide the necessary muscle mass, even without additional cell divisions before fusion. In addition, some myoblasts are set aside as individual adult progenitor cells, and will provide the source of cells for the adult muscles (Currie and Bate, 1991).

The cell adhesion molecule Dumbfounded (Duf; Kirre – FlyBase) is expressed exclusively in the founder cells. Duf has been suggested to provide an attractive cue for the FCMs, thereby mediating founder cell-FCM recognition and attachment (Ruiz-Gomez et al., 2000). Duf probably performs this function together with its paralog Roughest (Rst) (Strunkelberg et al., 2003), which has a less restricted expression pattern. Conversely, the Ig-domain proteins Sticks and stones (Sns) and Hibris (Hbs) are expressed by the FCMs, and through heterotypic interactions with Duf/Rst form the initial contact between the two cell types (Bour et al., 2000; Artero et al., 2001; Galletta et al., 2004; Shelton et al., 2009). Following Duf/Sns association, the fusion process ensues.

A large number of elements mediating myoblast fusion in *Drosophila* embryos has been identified (Abmayr and Pavlath, 2012). Many of these are known to function in the context of the actin-based cytoskeleton, and indeed, a large, transient actin ‘focus’ structure is present at sites of myoblast fusion (Kesper et al., 2007; Kim et al., 2007; Richardson et al., 2007; Gildor et al., 2009; Haralalka et al., 2011). Ultrastructural analysis has given rise to a number of models for progress of fusion at the subcellular level, in which roles for cell adhesion, vesicular entities and cytoskeletal structures have been proposed to provide a mechanistic basis for fusion pore formation and plasma membrane dissolution (Doberstein et al., 1997; Kim et al., 2007; Massarwa et al., 2007; Onel and Renkawitz-Pohl, 2009; Sens et al., 2010).

The process of multinucleated muscle formation is recapitulated during the pupal stages of *Drosophila* development, when nearly the entire larval musculature undergoes histolysis, and new adult muscles are generated (Sink et al., 2006). Similar to the embryo, most multinucleated adult muscle fibers are seeded by a founder cell, which fuses with neighboring myoblasts. An alternative

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myogenic program makes use of a few subsets of larval muscles that escape histolysis, and then provide templates for formation of adult muscles. Prominent among these is the process that generates the dorsal longitudinal indirect flight muscles (DLMs), a set of twelve large muscles housed within the adult fly thorax. In this case, a population of adult myoblasts positioned on the imaginal wing disc detach from the epithelium and migrate during early pupal stages toward a group of persistent thoracic larval muscles and fuse with them, leading to a significant enlargement of muscle mass (Fernandes et al., 1991).

Before and during the course of migration, these myoblasts divide, and can thus be regarded as ‘transit-amplifying cells’, before their fusion with the myotube templates. Another unique feature of these cells is their migration as a ‘swarm’ of closely associated cells, throughout their transit from the wing disc to the muscle templates (Roy and VijayRaghavan, 1998). It should also be noted that wing morphogenesis and other tissue movements in the developing pupa, generate a highly dynamic landscape in which these cells migrate. The underlying external cues that guide the migration of myoblasts, and the putative signals they may transmit to each other during the swarming process to maintain their transit-amplifying state, are not known.

Analysis of myogenesis during pupal stages has been limited by the difficulties in carrying out mutant clone analysis in a multinucleated tissue. We have previously shown that an RNAi-based approach can overcome these difficulties, in demonstrating the crucial involvement of *Wsp*, the sole fly homolog of WASp family actin nucleation-promoting factors, as well as other actin regulators, during adult myoblast fusion (Mukherjee et al., 2011).

Here we extend this approach to study the myogenic events preceding onset of fusion in DLM muscles, including association between myoblasts and DLM muscle templates, as mediated by cell-surface proteins, and the program of transcriptional regulation that endows myoblasts with a competence to fuse. In particular, we show that the *Duf/Rst* and *Sns/Hbs* pairs are essential for establishing the initial contact between myoblasts and myotubes. During migration as a swarm, we find that the myoblasts are maintained in the semi-differentiated, proliferating state, by continuous activation of Notch pathway signaling, where each myoblast provides the ligand Delta (*DI*) to its neighbors. Notch activity within the semi-differentiated myoblasts inhibits the ability of myoblasts to fuse, by repressing expression of key fusion-related factors such as *Sns* and the actin regulator D-WIP. Notch signaling between myoblasts decays once they reach the vicinity of the DLM myotubes, leading to terminal differentiation of the myoblasts, thereby enabling their productive association with myotubes and triggering the onset of fusion.

MATERIALS AND METHODS

Drosophila genetics

Expression of *UAS-dsRNA* was as described (Mukherjee et al., 2011). Where necessary, the *GAL80^{ts}/TARGET* system (McGuire et al., 2004) was used for temporal control of *UAS*-based transgene expression. *UAS-dsRNA* lines used: *Wsp* (GD13759); *Duf/Kirre* (GD3111 and KK109585); *Rst/IrreC* (GD27223 and JF03087); *Notch* (Bloomington line 7078 and JF02959); *DI* (GD3720 and JF02867); *Ser* (GD27172); *Sns* (GD877 and KK109442); *Hbs* (GD27065 and KK105913). GD and KK lines are from the Vienna *Drosophila* RNAi Center collection. JF lines are from the Harvard TRiP collection and were obtained from the Bloomington *Drosophila* Stock Center.

Tissue preparation and immunofluorescence

Dissected muscle preparations were obtained from staged pupae [collected as white prepupae at 0 hours after puparium formation (APF)]. Pupae of

the desired age (17–22 hours APF, unless otherwise specified) were removed from the pupal case, pinned down on Sylgard plates and dissected in cold PBS [see also Fernandes et al. (Fernandes et al., 1991)]. Fixation was commonly carried out with 4% paraformaldehyde (PFA) for 30 minutes, except for anti-Delta staining (see below). Following washes in PBS, the tissue was incubated with antibodies diluted in PBS+0.1% Triton-X and 0.1% bovine serum albumin as blocking reagent. Stained pupal preparations were mounted in 80% glycerol.

Primary antibodies used included: anti-GFP (chicken, 1:500, Aves), anti-Duf [rat, 1:1000 (Massarwa et al., 2007)], anti-Twist (rabbit, 1:5000, kindly provided by S. Roth, University of Cologne); anti-Vrp1/D-WIP [guinea pig, 1:500 (Berger et al., 2008)]; anti- β -galactosidase (mouse, 1:200, Promega); anti-Delta (monoclonal mouse, ascites fluid, 1:50, Hybridoma bank C594.9B); anti-Serrate (rat, 1:500, kindly provided by K. D. Irvine, Waksman Institute); anti-Sns [rabbit, 1:200 (Galletta et al., 2004)].

Secondary antibodies used included: Alexa Fluor 405 and Alexa Fluor 555 conjugated to rabbit anti-mouse antibodies (Molecular Probes) were used at a dilution of 1:500. Anti-mouse, rabbit, guinea-pig or rat antibodies conjugated to Cy2, Cy3 and Cy5 (Jackson ImmunoResearch) were diluted 1:400; Atto647N-Phalloidin (Fluka) was used at 2.5 μ g/ml; anti-chicken conjugated to DyLight 488 (Jackson ImmunoResearch) at 1:500.

PLP fixation protocol

For staining with anti-Delta antibodies, an alternative fixation protocol was used. Pupae of the desired age were removed from the pupal case, pinned down on Sylgard plates, dissected in cold PBS and fixed in 2% PFA, 1.35% lysine and 0.25% sodium periodate (NaIO₄) for 45 minutes. Cell membranes were permeabilized in PBS solution + 0.1% Saponin. All washes, primary and secondary antibody incubations were done in PBS + 0.1% saponin + 5% normal goat serum (NGS) (Hancock et al., 1982).

Clonal analysis

Larvae carrying *hs-FLP* and the *act-FRT-STOP-FRT-GAL4* cassette (Ito et al., 1997) were exposed to a mild heat shock at 34°C for 45 minutes, and allowed to age at 25°C. Pupae were timed and dissected as above. Analysis was performed on myoblasts that were adjacent to but not fully aligned with the myotubes, to avoid cells that have already begun the fusion process.

Image acquisition and processing

Images of immunofluorescent samples and live whole-mount samples were acquired using a Zeiss LSM 710 confocal scanning system, using $\times 10$ N.A 0.3, $\times 20$ N.A 0.5 or $\times 40$ N.A 1.1 lenses, and processed using Adobe Photoshop CS3.

RESULTS

Duf is required for adhesion of myoblasts to myotubes and establishment of fusion sites

During embryonic myogenesis *Duf* is expressed exclusively in founder cells and myotubes, and the protein is detected on the plasma membrane (Ruiz-Gomez et al., 2000; Menon and Chia, 2001). The *Duf* expression pattern, as revealed by expression reporters, is suggestive of myogenic roles during pupal stages as well (Guruharsha et al., 2009). To examine this issue further, we first used anti-Duf antibodies (Massarwa et al., 2007) to determine the *Duf* localization pattern during adult flight muscle formation. Within the developing DLMs (12–24 hours APF), *Duf* protein is found exclusively in myotubes, and cannot be detected in swarming myoblasts (supplementary material Fig. S1). At the subcellular level *Duf* resides mainly on the membrane of the myotubes, as well as in intracellular puncta (Fig. 1A; supplementary material Fig. S1). *Duf* localization on the myotube membrane is not uniform, but rather is enriched at contact points with fusing myoblasts (Fig. 1A'-A'''), as can be ascertained by colocalization with D-WIP, which marks myotube-myoblast contact sites (Mukherjee et al., 2011).

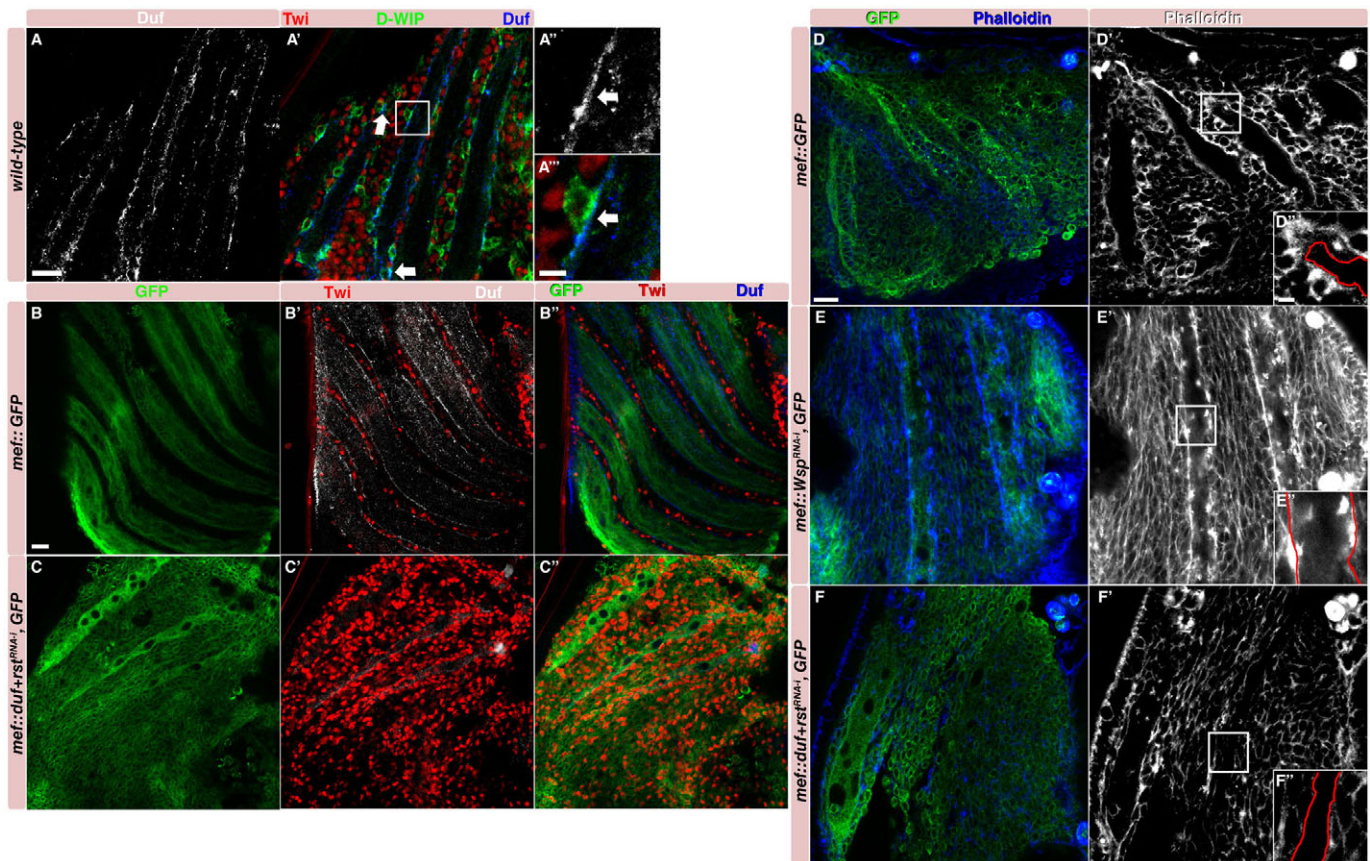


Fig. 1. Duf is expressed on myotubes and required for fusion. Dissected pupal muscle preparations, 18–22 hours APF. (A–A'') Staining for the Duf protein (white, blue) in control flies (*sns-lacZ/CyO*), demonstrating its specific expression only in myotubes but not in myoblasts. Myoblasts are identified via expression of the nuclear marker Twi (red), or D-WIP (green), which is upregulated in myoblasts just before fusion (Mukherjee et al., 2011), as Twi expression decays. The boxed region in A' is enlarged in A'' and A'''. Duf is enriched in myotube membrane domains adjacent to D-WIP expressing myoblasts (white arrows). (B–F) Muscle-specific RNAi knockdown phenotypes, generated by expression of different UAS-RNAi constructs via *mef2-GAL4*. UAS-CD8GFP (green) was expressed simultaneously, to visualize both myoblasts and myotubes. (B–B'') In control flies (*mef2-GAL4, UAS-CD8GFP, UAS-Dicer2/+*), where fusion occurs normally, the three DLM larval templates in each hemi-thorax grow in size and split, generating a set of six thick muscle fibers. Twi (red) stains only myoblasts, whereas Duf (white, blue) is specific to myotubes. (C–C'') Simultaneous *duf* and *rst* knockdown, achieved by combining the hypomorphic *duf* allele *duf^{p298}* (Nose et al., 1998), with expression of *duf* and *rst* RNAi constructs (*duf^{p298/Y}; duf^{RNAi} (GD3111), rst^{RNAi} (G272231)/+*; *mef2-GAL4, UAS-CD8GFP, UAS-Dcr2/+*), results in complete failure of the myoblasts to fuse with the DLM templates. Twi-positive myoblasts (red) remain clustered around the thin templates that also fail to split. Staining with anti-Duf protein (white, blue) demonstrates efficient elimination of the Duf protein. (D–F) Phalloidin (blue, white) was used to follow the formation of F-actin foci at the myotube/myoblast interface. GFP (green) marks all the myogenic cells. (D–D'') In control flies (*mef2-GAL4, UAS-CD8GFP, UAS-Dicer2/+*), the dynamic nature of the fusion process results in a small number of actin foci that form at the interface between fusing myoblasts and the myotubes. (E–E'') When fusion is blocked by *Wsp*-RNAi [*mef2-GAL4, UAS-CD8GFP, UAS-Dicer2/Wsp^{RNAi} (GD137591)*], numerous actin foci are observed, marking the sites of fusion arrest. (F–F'') By contrast, when fusion is blocked by *duf, rst* RNAi (*duf^{p298/Y}; duf^{RNAi} (GD3111), rst^{RNAi} (G272231)/+*; *mef2-GAL4, UAS-CD8GFP, UAS-Dcr2/+*), no actin foci are formed. Panels in this and all other figures display single, ~0.75 μ m thick optical sections, unless otherwise indicated. Scale bars: 20 μ m in A–F; 5 μ m in A''–F''.

The localization pattern of Duf is consistent with a role for this protein during DLM fiber development, possibly as a mediator of myoblast fusion with the fibers. RNAi-based knockdown of *duf* in myogenic tissue [using *mef2-GAL4* (Ranganayakulu et al., 1996)], did not affect DLM formation (supplementary material Fig. S2). Considerable abnormalities in the process of myoblast fusion became apparent, however, following simultaneous RNAi-based disruption of both Duf and Rst function (Fig. 1B–C''). Examination of thoracic muscles reveals that myoblast fusion to the myotubes is strongly arrested in this genetic background. This is observed by the marked reduction in myotube size, the failure of the splitting process that normally follows myoblast fusion, and in particular, by the small number of nuclei within the mutant myotubes, almost

all of which display the enlarged contours characteristic of polyploid larval template nuclei.

Further examination of the *duf, rst* knockdown fusion defect suggests that this phenotype results from a block of the fusion process at the initial phase of myoblast/myotube contact. We wanted to examine if cellular processes that take place after contact is established can be detected following *duf, rst* knockdown. Distinct actin foci have been shown to form at sites of myoblast fusion during adult muscle formation (Fig. 1D,D'), and persist in mutants such as *Wsp*, in which fusion arrests after myotube/myoblast contact is established (Mukherjee et al., 2011). We also observed numerous actin foci at the interface between DLM template myotubes and myoblasts following RNAi-based

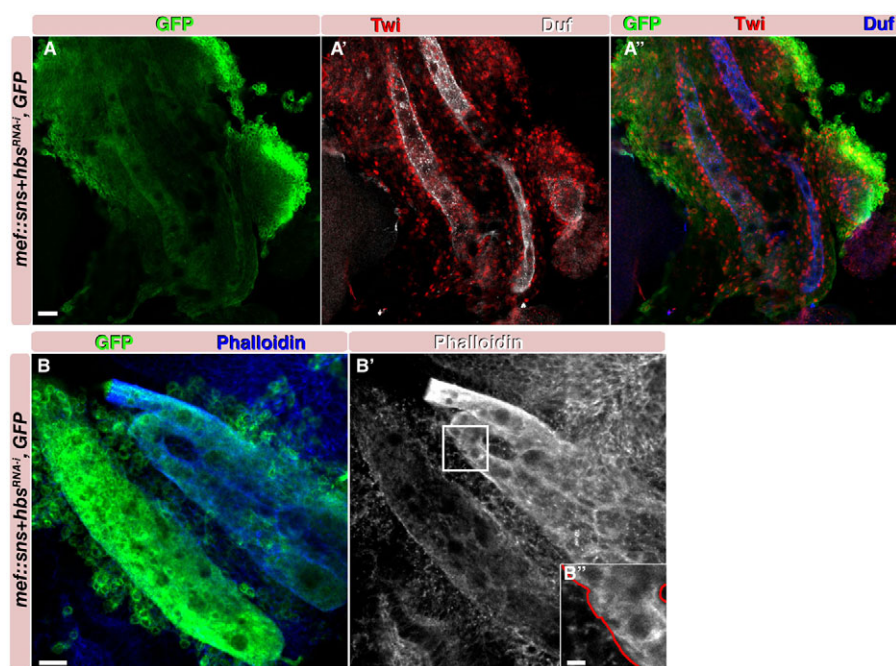


Fig. 2. Sns and Hbs are jointly required for myoblast fusion and formation of actin foci. Dissected pupal muscles 18–22 hours APF. Muscle-specific RNAi knockdown phenotypes were generated by expression of different UAS-RNAi constructs via *mef2-GAL4*. UAS-CD8GFP (green) was expressed simultaneously, to visualize both myoblasts and myotubes. **(A–A'')** When both *sns* and *hbs* are knocked down at the third instar larvae stage [*GAL80^{ts/+}; mef2-GAL4, UAS-CD8GFP, UAS-Dicer2/sns^{RNAi} (GD877), hbs^{RNAi} (GD27056)*], the myoblasts completely fail to fuse with the DLM templates. As a result, Twi-positive myoblasts (red) remain clustered around the thin templates (marked by Duf – white, blue), that also fail to split. **(B–B'')** Phalloidin staining (blue, white) reveals that fusion sites are not formed, as no actin foci are observed (compare with Fig. 1D,E). Scale bars: 20 μ m in A–B'; 5 μ m in B''.

Wsp knockdown (Fig. 1E,E'), but such foci were completely absent from *duf*, *rst* knockdown pupae (Fig. 1F,F'). Fusion sites are therefore not properly initiated in these pupae, suggesting incomplete contact between myotubes and myoblasts when *duf* and *rst* function is impaired.

Sns/Hbs function mediates establishment of myoblast-myotube fusion sites

Heterotypic interactions between the Duf/Rst and Sns/Hbs pairs of cell-surface proteins are generally accepted as the molecular basis for association between founder cells/myotubes and myoblasts in *Drosophila* embryos. *sns* mutant embryos display phenotypes very similar to *duf*, *rst* double mutants: dispersed myoblasts and mononucleated myotubes, indicating absence of adhesion between the FCs and founder cells, and a subsequent block to fusion between the two myogenic cell populations (Bour et al., 2000; Abmayr et al., 2003). Enhancement of these phenotypes, leading to complete fusion arrest, is observed in *sns*, *hbs* double mutant embryos (Menon et al., 2005; Shelton et al., 2009).

Separate knockdown of *sns* or *hbs* in pupae using RNAi constructs did not adversely affect flight muscle formation (supplementary material Fig. S2), but simultaneous knockdown of both elements resulted in a strong fusion arrest (Fig. 2). Similar to the phenotypes observed following *duf*, *rst* knockdown, DLM templates in the *sns*, *hbs* double knockdown pupae were small and unsplit, with a large population of myoblasts accumulated around them (Fig. 2A–A''). Furthermore, actin foci were completely absent from *sns*, *hbs* knockdown pupae (Fig. 2B–B''). Thus, while imaginal myoblasts reach the DLM myotubes following *sns*, *hbs* knockdown, fusion sites are not initiated, suggesting a block of the fusion process at the initial phase of myoblast/myotube contact.

Sns is expressed only in terminally differentiated adult myoblasts

To address the question of cell type requirement of the Sns/Hbs paralogs, we monitored the expression pattern of *sns* during DLM growth, using the expression reporter *sns-lacZ* (Kocherlakota et al.,

2008). Reporter expression, monitored by using anti- β -Gal antibodies, revealed that *sns* expression is restricted to a small subset of myoblasts positioned near the myotubes, but is absent from the bulk of the myoblast population, visualized using anti-Twist (Twi) (Fig. 3A–A''). This is in sharp contrast to the situation in the embryo, in which *sns* expression is detected in all fusion-competent myoblasts (Bour et al., 2000).

In addition to the limited myoblast expression pattern, *sns-lacZ* was detected in all DLM myotubes. This appears, however, to be an indirect consequence of incorporation of *sns-lacZ* expressing myoblasts into the templates, rather than a reflection of de novo myotube expression. This is supported by the observation that β -Gal staining is completely absent from myotubes of *Wsp* knockdown pupae, in which fusion is blocked (Fig. 3B).

The position of the *sns-lacZ* expressing cells in close vicinity to the myotubes suggested that they represent a late step in the gene-expression program of myoblasts. Indications of such a distinct late-stage expression program are also provided by the temporal expression pattern of Twi and D-WIP. Twi is expressed in the majority of myoblasts, yet it is downregulated in myoblasts just before fusion (Currie and Bate, 1991; Anant et al., 1998), whereas D-WIP is upregulated just before the formation of the actin foci and fusion (Mukherjee et al., 2011). Indeed, the subpopulation of *sns-lacZ* expressing myoblasts is mostly Twi-negative (~90%) and D-WIP-positive (~70%) (Fig. 3A,C).

The subcellular localization pattern of Sns within myoblasts neighboring flight muscle myotubes was determined using anti-Sns antibodies, which detect the protein at myoblast cell surfaces (Fig. 3E–E''). Double staining with anti-Duf reveals a striking correspondence between respective regions of Sns and Duf accumulation on myoblast and myotube cell membranes, implying that these proteins mediate contact and adhesion between the two cell types. Examination of similar preparations from pupae bearing the *sns-lacZ* reporter (Fig. 3F,G) demonstrates a close correspondence between the levels of *sns* reporter expression and Sns protein, supporting the notion of Sns induction just before fusion.

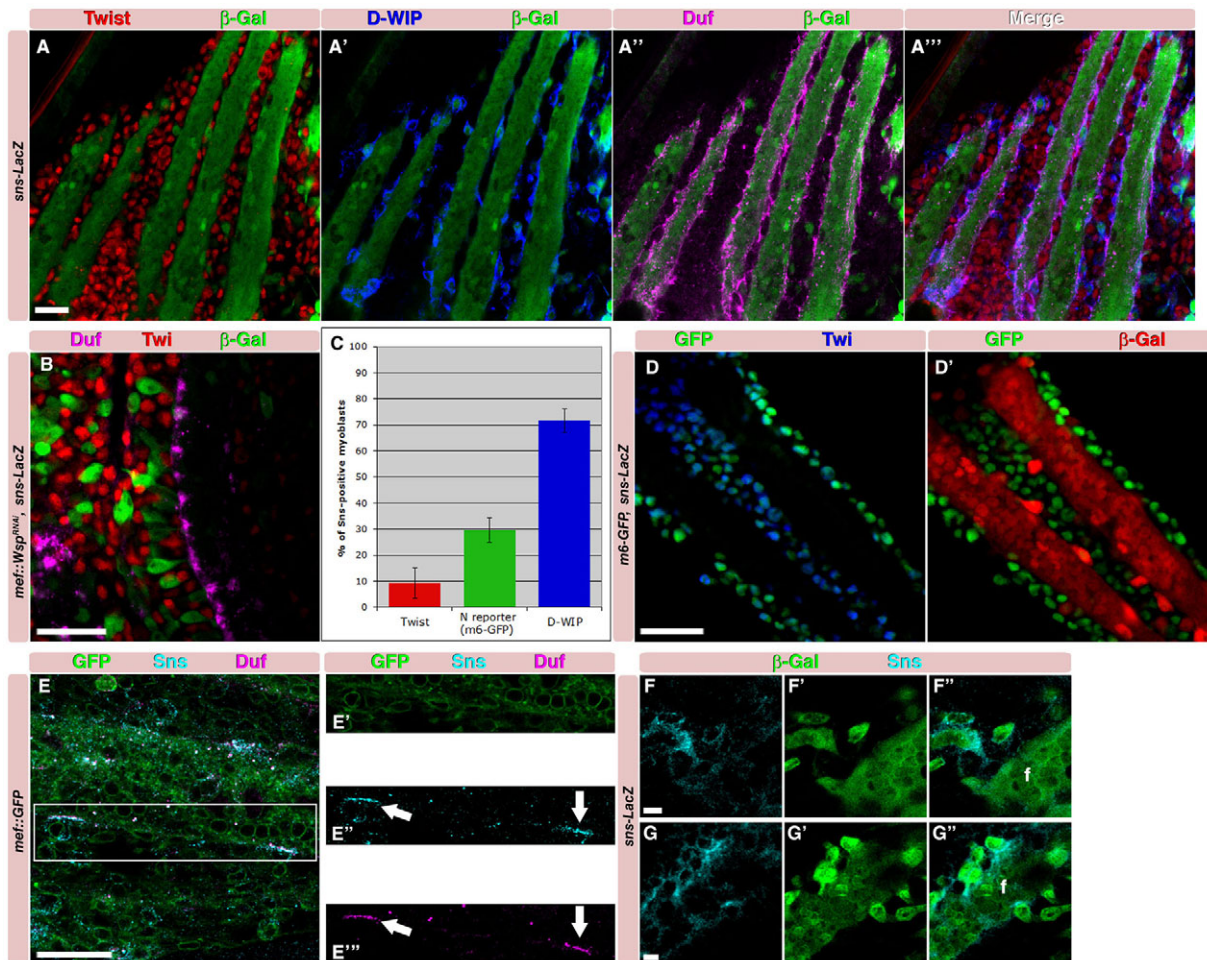


Fig. 3. Sns is expressed in a small subset of myoblasts. Dissected pupal muscles 18–22 hours APF. (A–A''') The Sns reporter *sns-lacZ* (green) reveals that Sns is expressed in myoblasts immediately adjacent to myotubes, and is co-expressed with D-WIP (blue), but not with Twi (red). Duf (magenta) marks the myotubes. (B) When fusion is blocked by using *Wsp^{RNAi}* [*GAL80^{TS}/sns-lacZ; mef2-GAL4, UAS-CD8GFP, UAS-Dicer2/Wsp^{RNAi} (GD13759)*], expression of Sns (green) is observed in Twi-negative myoblasts, but not in myotubes marked by Duf (magenta). This implies that *sns-lacZ* expression is restricted to myoblasts, and that its presence in myotubes (A–A''') is a consequence of fusion. (C) Quantification of the percentage of Sns-expressing myoblasts that co-express Twi (red, 9.3%±5.8, n=120), the Notch reporter *m6-GFP* (green, 29.6%±4.7, n=90) or D-WIP (blue, 71.6%±4.4, n=120). (D, D') Flies carrying both *sns-lacZ* (red) and the Notch reporter *m6-GFP* (green) display mutually exclusive expression patterns, and a positive correlation between the Notch reporter and Twi (blue). (E–E''') Anti-Sns antibody staining (cyan) reveals accumulations of Sns on myoblast surfaces, closely apposed to sites of Duf enrichment (magenta) on the myotube cell membrane (*mef2-GAL4, UAS-CD8GFP, UAS-Dicer2/+*). CD8-GFP (green) is driven by Mef2-GAL4, and therefore marks the membranes of both myotubes and myoblasts. E'–E''' show isolated views of the boxed area in E, highlighting these features. White arrows point to regions of Sns and Duf colocalization. (F–F''') Sns antibody staining of pupae carrying the *sns-lacZ* reporter (*sns-lacZ/+*) shows correspondence between high levels of the *lacZ* expression reporter (green) and high levels of Sns protein (cyan) in individual myoblasts. The myofibers are marked with 'f'. Scale bars: 20 μm in A–E; 5 μm in F, G.

Taken together, these observations imply a dynamic transcriptional program in wing disc-derived myoblasts, in which mature myoblasts lose some of their early markers, while initiating expression of elements necessary for fusion with the DLM templates. *sns* transcription is limited to this mature subpopulation of myoblasts. Such restriction and late onset of *sns* expression within the myoblast population suggests that a functional requirement for Sns commences only at the time of myoblast-myotube attachment. The *duf*, *rst* and *sns*, *hbs* double knockdown phenotypes, coupled with the Duf and Sns protein localization patterns, lead us to propose a sequence of events enabling fusion between wing disc-derived myoblasts and the DLM template myotubes. The process begins with the migration of myoblasts to the templates, and continues through adherence of myoblasts to

myotubes and establishment of fusion sites, mediated by Sns/Hbs-Duf/Rst interactions. These events 'set the stage' for execution of myoblast-myotube fusion, which will involve creation of F-actin foci at the fusion sites, fusion pore formation and membrane breakdown.

Notch signaling maintains the transit-amplifying state

Notch signaling has been implicated in the context of adult myogenesis in *Drosophila* (Anant et al., 1998; Bernard et al., 2006; Bernard et al., 2010). An interesting feature is that Notch signaling is active in most wing disc-derived myoblasts, but is shut down in a small number of those myoblasts that are in close proximity to the myotubes (Bernard et al., 2006). Using the Notch

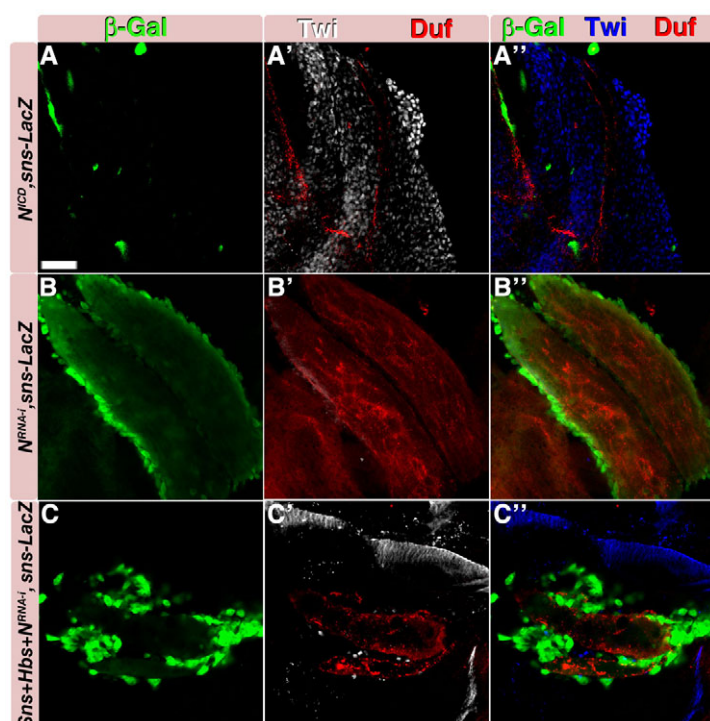


Fig. 4. Notch signaling represses Sns expression and myoblast fusion. Dissected pupal muscles 18 hours APF.

Expression of UAS constructs was restricted to metamorphosis using the *GAL80^{ts}/TARGET* system. **(A–A'')** In pupae in which the Notch pathway is constitutively active (*GAL80^{ts}/sns-lacZ*; *UAS-N^{ICD}*; *mef2-GAL4*, *UAS-CD8GFP*, *UAS-Dicer2/+*), accumulation of Twi-positive myoblasts (white, blue) around abnormally thin myotubes [marked by Duf (red)], indicates arrest of myoblast fusion. Expression of *sns-lacZ* (green) was completely blocked in myoblasts, but maintained in other tissues. **(B–B'')** Notch knockdown (*UAS-N^{RNAi} (7078)*; *GAL80^{ts}/sns-lacZ*; *mef2-GAL4*, *UAS-CD8GFP*, *UAS-Dicer2/+*) caused premature myoblast differentiation, marked by Sns induction and Twi repression, while fusion proceeded readily. **(C–C'')** When both Notch and *sns/hbs* are knocked down [*UAS-N^{RNAi} (7078)*; *GAL80^{ts}/sns-lacZ*; *mef2-GAL4*, *UAS-CD8GFP*, *UAS-Dicer2/sns^{RNAi} (GD877)*, *hbs^{RNAi} (GD27056)*], Sns-positive/Twi-negative myoblasts accumulated around the myotubes, but did not fuse with them. Background staining of non-myogenic tissue is a result of non-specific Twi antibody absorption. Scale bars: 20 μm in A–C''.

reporter line *m6-GFP* (Lai et al., 2000), we found that the myoblasts displaying reduced Notch signaling also show a decline in Twi levels (Bernard et al., 2006) (Fig. 3D). To test whether *sns* expression is linked to a reduction in Notch signaling, we examined *sns-lacZ* and *m6-GFP* expression in myoblasts of pupae harboring both reporters. Our analysis revealed that in most cases cells that upregulate Sns downregulated *m6-GFP* (~70%) (Fig. 3D,D', quantified in 3C). Perdurance of GFP induced by previous Notch activation is likely to make this figure an underestimate of the proportion of Sns-expressing cells in which Notch signaling is no longer active.

To investigate whether *sns* repression is indeed under the control of Notch, we chose to manipulate Notch signaling and monitor both myoblast fusion and the expression of *sns*. We first expressed the constitutively active intracellular domain of Notch (*N^{ICD}*) (Rebay et al., 1993) in both myotubes and myoblasts, under the potent *mef2-Gal4* driver. This resulted in a striking phenotype: expression of *sns* was completely absent from myoblasts, and fusion between myoblasts and DLM templates was blocked (Fig. 4A–A''). None of these dramatic effects can be elicited if expression of *N^{ICD}* is restricted to the myotubes (supplementary material Fig. S3), supporting the notion that the relevant Notch activities are myoblast-specific.

Next, we conducted the complementary experiment, generating a Notch loss-of-function setting specifically in the myogenic lineage. This was most efficiently obtained by expressing *Notch* RNAi via the *mef2-Gal4* driver. These results were also dramatic – not only was Sns expressed and Twi repressed in all myoblasts, but the size of the unfused myoblast population was noticeably small (Fig. 4B–B''); additional time points in supplementary material Fig. S4), suggestive of premature myoblast differentiation. To monitor the fate of the myoblast pool under conditions of *Notch* knockdown, we blocked fusion by further, simultaneous knockdown of *sns* and *hbs*. The triple knockdown resulted in a full arrest of fusion, and essentially all myoblasts were positive for Sns and negative for Twi expression (Fig. 4C–C'').

Taken together, the Notch gain- and loss-of-function experiments imply that Notch signaling suppresses the expression of Sns. Interestingly, Notch signaling appears to exert a positive, albeit mild, influence on expression of the Sns paralog Hbs (supplementary material Fig. S5) (see also Krejci et al., 2009). Only in mature myoblasts that are in close proximity to the myotube does a decline of Notch signaling enable induction of Sns expression, allowing for combined presence of both Sns and Hbs proteins in myoblasts only before the onset of fusion. We note that ectopic expression of *sns* cannot rescue the fusion-arrest phenotype associated with constitutive activation of Notch (supplementary material Fig. S6), suggesting that additional elements critical for myogenesis are similarly repressed by Notch signaling.

Delta-Notch signaling between myoblasts prevents premature differentiation

Notch signaling has been intensively studied in epithelial tissues, where the cells are stationary, and ligand-receptor interactions occur between permanent neighboring cells (reviewed in Artavanis-Tsakonas and Muskavitch, 2010). Migrating mesenchymal cells pose a challenge for canonical Notch signaling. Such cells can associate with a variety of neighbors along the way, and constantly alter their morphology. Thus, the dynamics of ligand sensing should adapt to these conditions. Since wing disc-derived pupal myoblasts migrate as a swarm, other cells in the swarm represent an obvious option for ligand presentation by neighboring cells.

We stained the swarming myoblasts and developing myotubes for the two Notch ligands D1 and Serrate (Ser). Both ligands are present on the myogenic cell membranes. Ser has a distinct discontinuous membrane pattern, mostly restricted to the myoblasts (Fig. 5A), whereas D1 shows a general membranal staining in both myoblasts and myotubes (Fig. 5B). We therefore used RNAi-based knockdown to assess the myogenic function of the two Notch ligands. Separate expression of RNAi constructs using the *mef2-GAL4* driver resulted in pronounced reduction in ligand epitope

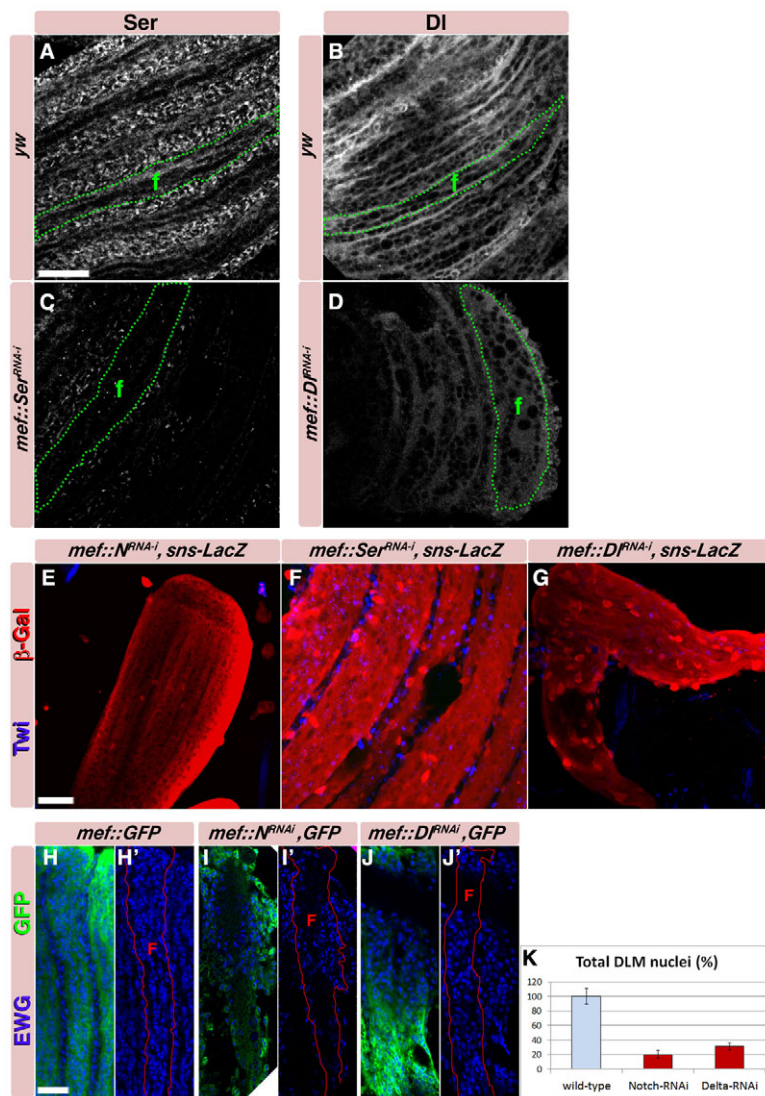


Fig. 5. Delta is the prominent ligand that signals to Notch in imaginal myoblasts. (A-D) Dissected pupal muscles 20 hours APF. Dotted outlines of single myotubes are marked with 'f'. (A,B) The Notch ligands Ser (A) and Df (B) are displayed on both myoblasts and myotubes in wild-type pupae. Ser appears to be enriched on myoblast membranes. (C) Ser RNAi (*GAL80^{TS}/sns-lacZ, UAS-Ser^{RNAi} (GD27172); mef2-GAL4, UAS-CD8GFP, UAS-Dicer2/+*) can efficiently knock down Ser levels in developing DLMs. (D) Df RNAi (*GAL80^{TS}/sns-lacZ, UAS-Df^{RNAi} (GD3720); mef2-GAL4, UAS-CD8GFP, UAS-Dicer2/+*) can efficiently knock down Df levels in developing DLMs. (E-G) Dissected pupal muscles 24 hours APF. (E) In Notch knockdown pupae (*UAS-N^{RNAi} (7078)+; GAL80^{TS}/sns-lacZ; mef2-GAL4, UAS-CD8GFP, UAS-Dicer2/+*) no Twi-positive myoblasts (blue) are present at this stage, and the few remaining unfused myoblasts are Sns-positive (red). (F) Pupae expressing Ser RNAi (*GAL80^{TS}/sns-lacZ, UAS-Ser^{RNAi} (GD27172); mef2-GAL4, UAS-CD8GFP, UAS-Dicer2/+*). Twi-positive cells are still present, similar to age-equivalent wild-type pupae. (G) Pupae expressing Df RNAi (*sns-lacZ/UAS-Df^{RNAi} (JF02867); mef2-GAL4, UAS-CD8GFP, UAS-Dicer2/+*). The vast majority of myoblasts have already fused, but only three muscle fibers are present. In the few remaining unfused myoblasts, Twi is repressed and *sns-lacZ* is expressed. (H-J') Dissected DLM muscles (22 hours APF) from pupae harboring the *mef2-GAL4* driver and UAS-CD8GFP, as well as UAS-RNAi constructs directed against Notch (H-I') or Delta (J-J'). The muscles were stained for GFP (green), to visualize myogenic cells, and for the nuclear marker Erect-wing (EWG) (blue). Single myofibers, marked with 'f', are outlined in the EWG-only panels. (K) Quantification of the number of EWG-positive nuclei within DLM fibers. EWG is expressed in myoblast nuclei as they reach the vicinity of the larval muscle templates, and following fusion, and can thus be used as a tool for quantitative assessment of the extent of fusion (Mukherjee et al., 2011). The total number of DLM nuclei in the different genotypes is shown, as a percentage of the control (*mef2-GAL4, UAS-CD8GFP, UAS-Dicer2/+*, 200±22 nuclei per fiber). The nuclear content of at least six fibers was determined for each genotype. Scale bars: 20 µm in A-J.

detection for both Ser (Fig. 5C) and Df (Fig. 5D). These observations demonstrate the capacity to interfere with Notch ligand expression and, furthermore, attest to the specificity of the antibodies used for ligand localization.

Reducing the levels of Ser by RNAi knockdown did not result in a prominent phenotype at 20 hours APF, i.e. the pupae possessed normal sets of split fibers, and the number of Twi-expressing myoblasts was comparable to those of age-matched wild-type pupae (Fig. 5F). However, reducing the levels of Df gave rise to dramatic phenotypes, reminiscent of those observed following knockdown of Notch. The DLM fiber set in these pupae is abnormally shaped and only partially split, and the small number of adhering, unfused myoblasts were all *sns-lacZ*-positive and Twi-negative (Fig. 5G; supplementary material Fig. S4). In addition, we determined that at this stage the fibers of both *Notch* and *Df* knockdown pupae contain far fewer nuclei than those of age-matched wild-type controls (Fig. 5H-K), further supporting the notion of premature incorporation of swarming myoblasts into the myofibers, before the myoblast population attained its full size. Taken together, these observations suggest that in the myoblast swarm, Notch activation levels are maintained predominantly via Df-Notch interactions between the myoblasts.

A fine balance between transmitting and receiving Notch signals in myoblasts

In a variety of systems where Notch signaling operates, the concomitant expression of ligand and receptor within the same cell results in 'cis-inhibition', where interaction between Notch ligands and receptors leads to inactivation of both partners. This feature was suggested to play a role in reducing signaling errors under conditions where stochastic activation of Notch patterns a tissue (Barad et al., 2010). As the migrating myoblasts express both ligand and receptor, we wanted to examine the possible contribution of cis-inhibition to Notch signaling in the myoblast swarm. By generating marked 'flip-out' clones (Basler and Struhl, 1994), we could specifically test the effect of elevated or reduced levels of either ligand or receptor, on the capacity of a cell to receive signals from its neighbors, while leaving the ability of the adjacent cells to present the signal intact. As myoblasts should dynamically exchange neighbors in the course of migration, altering the capacity of a small number of cells to send a signal should not have a significant effect on the entire population.

Flip-out clones marked by GFP expression were generated sporadically in myoblasts, by inducing an *hs-flp* construct at a relatively low temperature of 34°C. As a readout for Notch activation, we followed the capacity of the marked cells to express

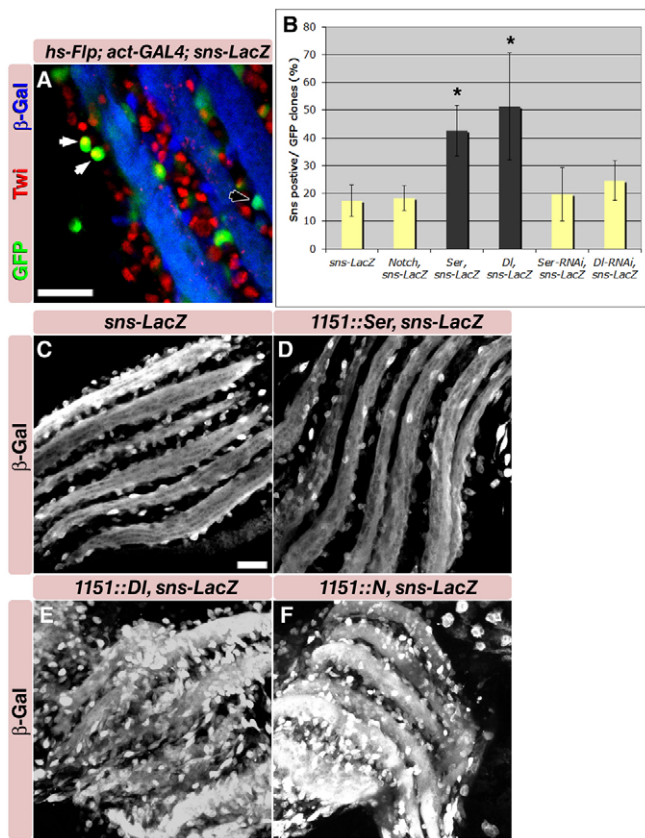


Fig. 6. High levels of Ser or Delta can cis-inhibit myoblast Notch signaling. (A) A mild heat shock at 34°C creates a ‘salt and pepper’ pattern of flip-out clone cells in the developing DLMs (*hs-Flp*, *UAS-GFP/+*; *sns-lacZ/CyO*; *act-FRT-STOP-FRT-GAL4/+*). Unfused myoblasts were monitored for the expression of GFP (green), *sns-lacZ* (blue) and Twi (red). White arrows indicate clone cells expressing both GFP and Twi; the black arrow indicates a clone cell expressing both GFP and Sns. (B) Percentage of *sns-lacZ* positive cells out of the total number of clone cells for each experiment. For each genotype the percentage was averaged from three to five different experiments. One hundred to three hundred clone cells were counted in total for each genotype. Asterisks denote the genotypes where a statistically significant deviation from the wild-type ratio was observed ($P < 0.05$). (C–E) Dissected pupal muscles 16–20 hours APF. Images are projections of 20–40 μm thick Z-stacks. (C) *sns-lacZ* expression in a wild-type background. (D) Overexpression of Ser (*1151-GAL4/+*; *UAS-Ser*; *sns-lacZ/+*) did not increase the fraction of *sns-lacZ* expressing myoblasts. (E, F) Overexpression of DI (*1151-GAL4/+*; *UAS-DI*; *sns-lacZ/+*) (E) or of the N receptor (*1151-GAL4/+*; *UAS-N*; *sns-lacZ/+*) (F), significantly increased the fraction of *sns-lacZ* expressing myoblasts (from 12 to 35% of the total myoblast (Twi-positive + *lacZ*-positive) population in both genotypes). Scale bars: 20 μm .

Twi (Notch on) or *sns-lacZ* (Notch off). The fraction of *sns-lacZ*-expressing myoblasts in the control flip-out cells or following co-expression of different constructs was monitored (Fig. 6A). We first induced the expression of Notch in the flip-out cells. Although this manipulation may titrate the ligand displayed by these cells, it should not compromise their capacity to receive a signal from neighboring cells. Indeed, no alteration in the fraction of *sns-lacZ*-positive cells was observed (Fig. 6B). By contrast, elevation of Notch ligand levels led to an increase in the fraction of *sns-lacZ*-positive cells, with a more pronounced effect when DI was

overexpressed (Fig. 6B). These results indicate that elevation in the levels of Ser or DI compromises the capacity of the same cells to receive a signal from neighboring cells, presumably by cis-inhibition, which titrates the receptor.

To monitor the consequence of a global perturbation in the balance of ligand and receptor, Ser or DI were overexpressed in all myoblasts, using the myoblast-specific *1151-Gal4* driver (Roy and VijayRaghavan, 1997). Global myoblast Ser overexpression did not give rise to a marked elevation in Notch signaling, as reflected in the fraction of *sns-lacZ*-expressing myoblasts (Fig. 6C,D). Conversely, DI overexpression gave rise to a phenotype that was reminiscent of DI or Notch knockdown (albeit weaker): a significantly larger fraction of myoblasts ($35 \pm 10\%$ versus $12 \pm 2\%$ in wild type) expressed *sns-lacZ* (Fig. 6E). Thus, despite the presentation of more ligand by all cells, the predominant outcome was a reduction in Notch signaling, due to cis-inhibition of the receptor. Overexpression of the Notch receptor in all myoblasts similarly raised the fraction of *sns-lacZ*-expressing myoblasts (Fig. 6F) ($35 \pm 8\%$ versus $12 \pm 2\%$ in wild type). In this case we envision that excess receptor sequesters the ligand presented by the myoblasts. This observation, which is consistent with the cis-inhibition scenario, harbors additional significance, as it implies that myoblasts are the exclusive source of ligand triggering Notch activation.

Overexpression in myoblast clones or in the whole myoblast population has demonstrated that higher levels of Ser or DI can perturb the balance of Notch signaling. Are the endogenous levels of DI and Ser fine-tuned and set at a range that is below the cis-inhibition threshold? Expression of RNAi for Ser or DI in individual myoblast cells should allow detection of alterations in their capacity to receive the Notch signal, without altering the overall ligand-mediated signaling capacity of the myoblast population. No significant alteration in the receiving capacity of the flip-out cells was observed, when RNAi constructs for Ser or DI were expressed (Fig. 6B). Thus, the endogenous Ser and DI levels are set such that they provide effective signaling between cells, but do not compromise the reception capacity.

Taken together, our findings indicate that the levels of ligand and receptor co-expressed by the swarming myoblasts are finely tuned, to maintain the capacity of these cells to present and receive signals simultaneously during their migration. Proper continuous levels of Notch signaling are essential in order to sustain the swarming myoblasts in the proliferative transit-amplifying state, until they encounter the DLM myotube templates.

DISCUSSION

Myogenesis in the *Drosophila* pupa, culminating in formation of the adult musculature, presents a variety of challenges for faithful execution of the process (Roy and VijayRaghavan, 1999). Here we studied the formation of the indirect DLM flight muscles, where myoblasts face the task of reaching and fusing with larval templates. In the course of ~24 hours after pupariation, these wing disc-derived myoblasts undergo continuous proliferation, in order to generate a sufficiently large pool of cells that will eventually contribute to the adult muscle mass. The myoblasts progress through two distinct phases of differentiation during the process: a semi-differentiated, amplifying phase during the course of swarming; and a terminally differentiated, fusion competent phase, once within close range of the myotubes. This program differs in key aspects from myogenesis in the *Drosophila* embryo, a setting in which events preceding and during myoblast fusion have been thoroughly studied. Thus, embryonic FCMs, which are mostly

postmitotic, traverse relatively short distances toward founder cells and myotubes, and appear to behave as individual migratory cells rather than as a group (Beckett and Baylies, 2007). Correspondingly, we find both similarities and fundamental differences in the manner by which fusion-related elements are employed and regulated in the two myogenic systems.

In the current study, we show that mutual bidirectional signals within the myoblast swarm maintain the semi-differentiated fate of the migrating cells. In this novel and dynamic microenvironment, the myoblasts continuously exchange neighbors, yet most of the time these neighbors are identical to each other. Inhibition of the transcriptional program governing the migratory phase, and onset of gene expression necessary for full differentiation and fusion, are likely to result from short-range, myotube-derived signals that the myoblasts encounter, once they reach the vicinity of the myotubes. Our interpretation of the data reported in this study is summarized in the model shown in Fig. 7.

Our analysis of myoblast fusion identifies the pairs of Ig-domain, cell-surface proteins Duf/Rst and Sns/Hbs, as key elements mediating the attachment of myoblasts to DLM myotube templates. Redundancy in the function of these protein pairs has been described during myogenesis in the *Drosophila* embryo (Ruiz-Gomez et al., 2000; Shelton et al., 2009), and appears to be a feature of the program underlying formation of adult muscles as well. One apparent difference between the embryonic and pupal settings is in regulation of the myoblast-specific element Sns, whose expression in the pupae is restricted to the period just before fusion.

Although it has been previously shown that myoblasts reaching the myotube change some of their molecular markers, the full significance of this process in the context of myoblast-myotube fusion has not been elucidated. Our results suggest that the myoblasts sense a continuous Notch signal, from the time the cells detach from the wing disc and even earlier, until the moment they reach the myotube vicinity. This signal appears to have a dual role, maintaining the cells in a proliferative mode, as well as preventing them from obtaining the competence to fuse. Notch suppression of *sns* and other fusion-related genes such as *D-WIP* therefore prevents the untimely development of muscles. Simultaneous suppression of a battery of fusion-related genes by Notch is corroborated by our inability to rescue the *Notch* knockdown phenotype by ectopic expression of Sns alone.

Many features of pupal myogenesis resemble the repair of injured adult muscles in vertebrates. Stem cells, termed satellite cells, are located within the muscle in close proximity to the basal lamina. They undergo an asymmetric division, where one progeny leaves its original site and undergoes proliferation, to generate a transit-amplifying population. These dividing, semi-differentiated cells do not display the full expression profile of mature myoblasts. The proliferative phase requires continuous activation of Notch, and relieves the stem cells from the need to divide asymmetrically at a mode where only a single differentiated myoblast is created at a time. The cells gradually shift to the fully differentiated fate, first in only one progeny of each division, and eventually in all cells. The differentiated cells are capable of fusing with the injured muscle to increase its mass (reviewed by Luo et al., 2005). We suggest that, by analogy, the swarming *Drosophila* pupal myoblasts that are defined by Notch activation and *Tw* expression possess a 'transit-amplifying' character, not yet ready to undergo fusion to the myotubes.

Notch signaling in swarming pupal myoblasts is unique, in the sense that the same cell simultaneously sends and receives the

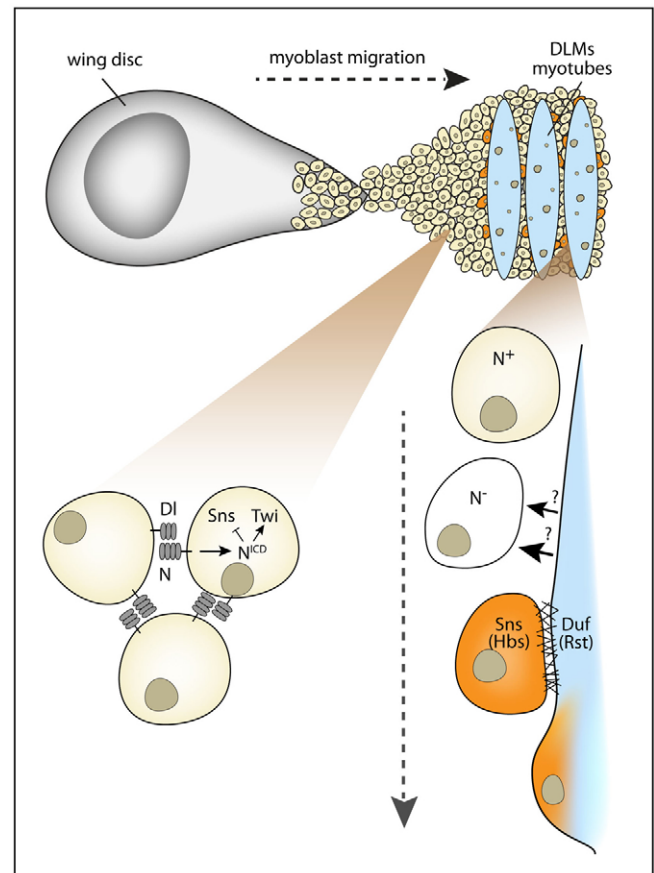


Fig. 7. Notch signaling represses Sns expression and fusion competence in migrating myoblasts.

Myoblasts that were set aside during embryogenesis to serve as adult precursors for the indirect flight muscles are positioned on the wing imaginal disc, at the region that will give rise to the notum. During pupariation these cells proliferate and migrate as a swarm toward the DLM muscle templates, with which they eventually fuse. The myoblasts are maintained in a precursor state by continuous activation of Notch signaling, triggered by exposure to DI signals emanating from the adjacent myoblasts in the swarm. Notch activation induces expression of *Tw*, and suppresses expression of genes important for fusion such as *Sns*. We suggest that an unknown signal secreted from the myotube, and operating only at close vicinity, leads to termination of Notch signaling in the approaching myoblasts. The induced expression of *Sns* and additional fusion-related elements in the myoblasts adjacent to the myotubes, reflects their terminal differentiation. Attachment of myoblasts to myotubes (mediated by the cell-surface proteins Duf/Rst and Sns/Hbs) and fusion between the two myogenic cell types will then ensue, providing for flight muscle growth.

signal, over a prolonged time interval. The stochastic contact of the myoblasts with each other as they transit may allow them to undergo repeated cycles of Notch activation, each time triggered by exposure to a different ligand-presenting cell. A given, albeit fluctuating level of Notch signaling is thus maintained, to preserve the cells in a proliferative, semi-differentiated state, until they encounter the myotube. These observations highlight the unique nature of the myoblasts in their migratory phase. Interestingly, in contrast to the repeated activation of Notch in all swarming myoblasts, a mechanism utilizing the transient nature of interactions between migrating cells has recently been described for the chick somites (Rios et al., 2011). Migrating neural crest

cells express Delta1 in a mosaic pattern, and although all somitic cells express the receptor, Notch activation is restricted to those cells that transiently encounter the Delta1-positive neural crest cells passing by.

In our analysis, knockdown experiments have shown that DI is the functional ligand presented by the myoblasts in the context of the pupal myoblast swarm. Mosaic experiments have shown that elevation in the levels of DI or Ser expressed by the myoblasts compromise the capacity to receive signals, presumably by cis-inhibition of the receptor. The levels of ligands and receptor expressed by each myoblast thus need to be finely tuned, so that each cell maintains its ability to both send and receive signals. A similar scenario was described during the maturation of the immune synapse. The allogeneic recognition between Th cells and dendritic cells is mediated by bidirectional Notch signaling between the cells. In this case, cis-inhibition is prevented by physical segregation of ligands and receptor within the same cell. In one cell type the ligands are at the center and the receptor is at the periphery of the immune synapse, whereas in the opposing cell they display a complementary distribution (Luty et al., 2007).

Once the myoblasts are in close proximity to the myotubes, Notch signaling is terminated. This leads to shutdown of Twi, and induction of Sns, D-WIP and probably a battery of additional genes that are required to execute the fusion process. We assume that the signal(s) to terminate Notch signaling originate from the myotubes, as myoblasts that shut off the Notch reporter and turn on Sns are typically observed in close proximity to the myotubes. The nature of these putative short-range signals terminating Notch signaling is not known. In the satellite cell system, it was suggested that Wnt signaling emanating from the myotube terminates Notch signaling in the transit-amplifying cells (Brack et al., 2008). Whether this is also true during the formation of the adult flight muscles in *Drosophila*, remains to be seen. However, regardless of the identity of these molecular mechanisms, considerable similarities in the myogenic programs governing development of the adult fly and vertebrate musculature are clearly evident.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.077495/-/DC1>

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