Newly identified satellite cells respond to damage through Notch-Delta signaling to fuse with adult *Drosophila* muscles.

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Abstract

In previous work, we identified muscle-specific stem cells, which asymmetrically divide to give rise to myoblasts, which fuse to form flight muscle fibres in Drosophila (Gunage et al., 2014). Here, we show the presence of lineal descendants of these stem cells as small, un-fused cells located superficially and in close proximity to mature adult muscle fibres. Normally quiescent, these cells become mitotically active following muscle injury. In view of their strikingly similar morphological and functional features, we consider these cells to be the *Drosophila* equivalent of vertebrate muscle satellite cells. Thus, in flies as in vertebrates, the stem cell lineage that generates adult-specific muscles is also available for myogenesis in mature muscle. The mechanism of satellite cell activation uses Delta in the muscle fibre signaling to Notch in satellite cells. These results demonstrate the mechanistic value of the fly for further studies on muscle regeneration.

Introduction

A great deal of insight into the cellular and molecular mechanisms of muscle development has been obtained in two powerful genetic model systems, namely the mouse and *Drosophila*. Despite numerous differences in the specific ways in which muscles are formed in these two organisms, there are also remarkable similarities in the fundamental developmental processes that underlie myogenesis (Roy and VijayRaghavan, 1999; Sink, 2006; Rochlin et al., 2010). These similarities are also evident when the mechanisms of myogenesis of the large multi-fibrillar indirect flight muscles of *Drosophila* are compared to

vertebrate skeletal muscles. In both cases, muscle stem cells generated during embryogenesis give rise to a large pool of muscle precursor cells called myoblasts that subsequently differentiate and fuse to produce the multinucleated syncytial cells of the mature muscle (Dequéant et al., 2015; Gunage et al., 2014; Sambasivan and Tajbakhsh, 2007). These mechanistic similarities of myogenesis are reflected at the molecular genetic level, in that many of the key genes involved in *Drosophila* muscle development have served as a basis for the identification of comparable genes in vertebrate muscle development and vice-versa (Srinivas et al., 2007; Schnorrer et al., 2010; Relaix and Buckingham, 2010; Abmayr and Pavlath, 2012).

In vertebrates, mature skeletal muscle cells can manifest regenerative responses to insults due to injury or degenerative disease. These regenerative events require the action of a small population of tissue specific stem cells referred to as satellite cells (Brack and Rando, 2012; Relaix and Zammit, 2012). Muscle satellite cells are located superficially in the muscle fibers and surrounded by the basal lamina of the fibers. Although normally quiescent, satellite cells respond to muscle damage by proliferating and producing myoblasts, which differentiate and fuse with the injured muscle cells. Myoblasts generated by satellite cells are also involved in the growth of adult vertebrate muscle. Given the numerous fundamental aspects of muscle stem cell biology and myogenesis that are similar in flies and vertebrates, it is surprising that muscle satellite cells have not been reported in *Drosophila*. Indeed, due to the apparent absence of satellite cells in adult fly muscles, it is unclear if muscle regeneration in response to injury can take place in *Drosophila*.

In a previous study, we showed that a small set of embryonically generated muscle-specific stem cells known as AMPs (adult muscle

progenitors) give rise postembryonically to the numerous myoblasts which fuse to form the indirect flight muscles of adult *Drosophila* (Gunage et al., 2014). Here we investigate the fate of the muscle stem cell-like AMPs in the adult by clonal tracing analysis and find that lineal descendants of these muscle stem cells have all of the anatomical features of muscle satellite cells. In adult muscle they remain unfused, located in close proximity to the mature muscle fibers and are surrounded by the basal lamina of the fibers. Moreover, although normally quiescent, they retain the potential to become mitotically active and undergo Notch signaling-dependent proliferation following muscle injury. In view of these remarkable developmental, morphological and functional features, we consider these cells to be the *Drosophila* equivalent of vertebrate muscle satellite cells. Thus, in flies and vertebrates the muscle stem cell lineage that generates the adult-specific muscles during normal postembryonic development is also available for adult myogenesis in muscle tissue in response to damage.

Material and Methods

Fly strains, genetics and MARCM

Fly stocks were obtained from the Bloomington Drosophila Stock Centre (Indiana, USA) and were grown on standard cornmeal medium at 25°C.

For MARCM experiments flies of genotype Hsflp/Hsflp; FRT 42 B, Tub Gal80 were crossed to flies of genotype +; FRT 42 B, UAS mCD8::GFP / Cyo Act-GFP ; Dmef2-Gal4 or +; FRT 42 B, UAS nls::GFP / Cyo Act-GFP ; Dmef2-Gal4. For MARCM experiments, two heat shocks of 1h each separated by 1h were given to either late third instar larvae or young

adults for clonal induction. In both cases recovery of labeled clones was in adult stages, which were dissected and processed for flight muscles. In knockdown and overexpression experiments the following lines were used: +; +; Dmef2-Gal4, Gal80ts, Act 88F-Gal4, Gal80ts, UAS Notch RNAi (Bloom, 35213), UAS NICD, UAS DN Delta, UAS Delta RNAi (Bloom, 37288 and GD3720). Other fly stock used was w1118; P{NRE-EGFP.S}5A.

Immunohistochemistry and confocal microscopy

Flight muscles were dissected from specifically staged (1-10 day old) flies and then fixed in 4% paraformaldehyde diluted in phosphate buffered saline (PBS pH-7.5). Immunostaining was performed according to (Hunt and Demontis, 2013) with few modifications. In brief, samples were then subjected to two washes of 0.3% PTX (PBS + 0.3% Triton-X) and 0.3% PBTX (PBS + 0.3% Triton-X + 0.1 %BSA) for 6h each. Primary antibody staining was performed for overnight on a shaker and secondary antibodies were added following four washes of 0.3% PTX 2h each. Excess of unbound secondary antibodies was removed at the end of 12h by two washes of 0.3% PTX 2h each following which samples were mounted in Vectashield mounting media. For immunostaining, anti-NICD (Notch intracellular C-terminal domain) (Mouse, 1:100, DSHB), anti-GFP (Chick, 1:500, Abcam, Cambridge, UK), anti-Delta (monoclonal mouse, 1:50, Hybridoma bank C594.9B), anti-MHC (Mouse, 1:100, kind gift from Dr. Richard Kripps), TOPRO-3-Iodide (1:1000, Invitrogen), anti-Neuralised (1:50, Rabbit)(Lai et al., 2001), phalloidin (Alexa-488 and rhodamine conjugate, 1:500, ThermoFisher), anti-phosphohistone-3 (Rabbit, 1:100, Millipore) antibodies were used. Secondary antibodies (1:500) from Invitrogen conjugated with Alexa fluor-488, 568 and 647 were used in immunostaining procedures.

Confocal and electron microscopy

For confocal experiments, an Olympus FV 1000 confocal point scanning microscope was used for image acquisition. Images were processed using ImageJ software (Rasband WS, ImageJ U S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2012). Quantification of number of actively dividing cells in PH-3 labeling experiments was performed as described in (Gunage et al., 2014).

For electron microscopic analysis the muscles were processed according to Garcia-Murillas et al., 2006. In brief, flight muscles were dissected in ice-cold fixative (2.5% glutaraldehyde in 0.1 M PIPES buffer at pH 7.4). After 10hrs of fixation at 4°C, samples were washed with 0.1M PIPES, post-fixed in 1% OsO4 (30min), and stained in 2% uranyl acetate (1hr). Samples were dehydrated in an ethanol series (50%, 70%, 100%) and embedded in epoxy. Ultrathin sections (50 nm) were cut and viewed on a Tecnai G2 Spirit Bio-TWIN electron microscope.

Muscle injury

To induce regeneration response in the flight muscle, we developed injury assay. For this, flies aged for 1, 3, 5 and 10 days were used. Flies were CO_2 anaesthetized and a single stab injury was performed manually with dissection pin or tungsten needle. Care was taken so that the tungsten needle tip did not cross the hemithorax so that the damage was restricted to a minimum area. The anatomical location of the injury was as shown in figure 3A. Control flies were age matched adult flies but with no injury to muscles. After injury, a recovery period of 12 h on corn meal *Drosophila* food was given. The flies were then processed for

immunostaining of flight muscles as mentioned in the immunohistochemistry procedure.

Results

Cells of the adult muscle precursor (AMP) lineage persist unfused in adult muscle.

During normal postembryonic development of the indirect flight muscles, a set of approximately 250 mitotically active AMPs located on the epithelial surface of the wing imaginal disc generates a large number of postmitotic myoblast progeny. These myoblasts subsequently migrate and fuse with larval templates to produce the mature muscle fibers of the adult (Gunage et al., 2014; Dhanyasi et al., 2015). Whether or not their AMP progenitors persist in the adult and also interact with the mature muscle fibers is unknown (Figure 1A).

To investigate this, we used MARCM cell-labeling methods (Wu and Luo, 2006) to induce clonal labeling of the muscle stem cell lineages in late larval stages and recovered labeled clones in the adult muscle. In this MARCM analysis, Dmef2-Gal4 was used to express a mCD8::GFP reporter; labeled cells in the adult were visualized using confocal microscopy. If all the stem cell-like AMPs similar to their postmitotic myoblast progeny fuse with the adult muscle then no mCD8::GFP labeled cells should be visible in the adult flight muscles (following fusion, membrane tethered GFP becomes diffuse due to incorporation into the extensive muscle fiber membrane). In contrast, if AMP lineal descendants do persist as unfused cells in the adult muscles, then individual, mono-nucleated cells that have their entire membrane labeled

with GFP should be observed and such unfused cells are indeed seen (Fig. 1B).

Furthermore, and strikingly, this clonal analysis reveals the presence of numerous unfused, mCD8::GFP-positive mononucleated cells closely apposed to the surface of the adult muscle fibers (Fig. 1C-E). These small wedge-shaped labeled cells are distributed along the entire surface of the muscle fibers and are located both at the interface between different muscle fibers and at the surface of individual muscle fibers (Fig. 1F-I). These findings indicate that a set of cells of the AMP lineage which differ from those myoblasts which fuse, persist in the adult muscle. Moreover, they show that these AMP lineal descendant cells, in contrast to myoblasts, do not fuse with the mature muscle cells but remain unfused, albeit closely associated with the muscle fibers in the adult.

Unfused muscle-associated cells have ultrastructural features of satellite cells

In terms of their arrangement, size and lineal origin, these unfused, adult muscle-associated cells are comparable to the satellite cells found in mature vertebrate muscle. To investigate this similarity further, we carried out an electron microscopic analysis of adult indirect flight muscle fibers in the fly.

In electron-micrographs, mature muscle fibers are large cells containing multiple prominent nuclei, numerous organelles, as well as extensive sets of elongated myofibrils, and are surrounded by a prominent extracellular matrix (Fig. 2A, B). In addition to these typical muscle cells, the ultrastructural analysis revealed the presence of small, wedge-shaped unfused cells located superficially and closely apposed to the large multinucleated muscle fibers. These small cells have compact nuclei and

small cytoplasmic domains with few organelles. The intact cell membrane of these cells is directly adjacent to the muscle cell membrane and embedded in the extracellular matrix of their adjoining muscle fiber (Fig 2B).

Morphologically, the small unfused cells in adult muscle have many similarities to vertebrate satellite cells. In both cases, the cells are small, mononucleated and intercalated between the cell membrane and the extracellular matrix of mature muscle fibers. Based on their anatomical similarities with vertebrate satellite cells and considering their comparable lineal origin from muscle stem cells, we posit that these small, unfused muscle fiber-associated cells are the insect equivalent of vertebrate muscle satellite cells.

Muscle injury results in proliferative activity of Drosophila satellite cells

Vertebrate satellite cells are essential for muscle regeneration and repair; muscle damage results in mitotic activity of satellite cells and the proliferative production of myoblasts that rebuild compromised muscle tissue. To investigate if the muscle satellite cells in *Drosophila* can also respond to muscle injury by proliferative activity, we induced physical damage in adult flight muscles mechanically (Fig. 3A, C). Subsequently, we probed the damaged muscle for cells showing mitotic activity using the mitotic marker phosphohistone-3 (PH-3).

In undamaged controls, PH3-labeled cells were rarely observed (Fig. 3B). In contrast, numerous PH-3 labeled cells were seen in muscles 12h after physical damage had been induced (Fig. 3C). A quantification of the number of PH-3 labeled cells in control versus damaged muscles shown

underscores this fact (Fig. 3D). (Similar results were obtained in experiments that used EdU labeling to monitor cell cycle entry; data not shown.) These findings indicate that physical damage leads to a pronounced increase in proliferative activity in cells associated with the flight muscle. Importantly, the labeled cells in the damaged muscle were generally intercalated between muscle fibers implying that they correspond to the satellite cells.

To confirm this, we performed lineage tracing with MARCM methods by inducing clonal labeling of the AMP lineages in late larval stages and recovering mCD8::GFP-labeled clones in the adult muscles 24h after physical damage. Strikingly, virtually all of the individual cells in the membrane tethered GFP-labeled clones were PH-3 positive implying that these AMP lineal descendants were mitotically active in the damaged muscle (Fig. 3E-H). Moreover, based on the membrane tethered nature of the GFP labeling, at least a subset of the PH3-labeled cells was not fused with the muscle fibers, again implying that they correspond to satellite cells.

If AMP lineal descendants present as satellite cells in mature muscle become mitotically active following injury, might some of the progeny they generate in the adult correspond to myoblast-like cells that fuse with the damaged muscle? To investigate this, we used MARCM methods to visualize the lineal progeny of the mitotically active cells in injured adult muscle. Clones were induced in the adult 6h prior to physical injury and nls-GFP labeled clones were recovered 24h later. In these experiments, nls-GFP labeled nuclei were seen both outside and inside of the muscle fiber implying that a subset of the labeled cells had fused with the mature muscle (Fig. 3I-N). These findings indicate that AMP lineal descendants

present as satellite cells in adult muscle can proliferate following muscle injury and generate daughter cells that fuse with the muscle fibers (Fig. O).

Activation of Drosophila satellite cell proliferation is controlled by Notch signaling

Previously we have shown that proliferative mitotic activity of AMPs during development requires Notch signaling (Gunage et al., 2014). Might the AMP lineal descendant satellite cells in adult muscle also require Notch signaling for injury-induced mitotic activity? Immunolabeling of satellite cells co-labeled by MARCM lineage tracing shows that these cells do express Notch (Fig. 4A-C). To determine if Notch expression in these cells is required for their mitotic activity following muscle injury, we used a temperature sensitive Notch allele together with PH-3 labeling. While numerous muscle-associated cells were PH-3 labeled at the permissive temperature implying that functional Notch is indeed required for satellite cell proliferation, at the restrictive temperature only few cells are PH-3-positive (Fig. 4D). This was confirmed in Dmef2-driven Notch-RNAi knockdown experiments, which results in a dramatic reduction of PH-3 labeled cell number in injured muscles (Fig. 4E-G). Moreover, an assay of canonical Notch signaling using an NRE-GFP line (Notch Responsive Element, a GFP fusion construct of E(spl); Saj et al., 2010) shows a marked increase in labeling of muscle associated cells in injured muscle compared to uninjured controls (Fig. 4H-N). These results indicate that Notch signaling is required in muscle-associated satellite cells for their proliferative mitotic activity in injured muscle.

Immunolabeling shows that the Notch ligand Delta is expressed widely in normal as well as injured flight muscle fibers (Fig. 4O, P; data not shown). This suggests that Delta in muscle fibers might be required to activate Notch signaling in satellite cells following muscle damage. In accordance with this, in Act88F-driven (muscle-specific) Delta-RNAi knockdown experiments a marked reduction in the number of PH-3 labeled muscle associated cells in injured muscles was observed as compared to controls (Fig. 4 O-Q). Similar findings were obtained when a dominant negative form of Delta was expressed in injured muscle fibers (Fig. 4Q). Interestingly, immunolabeling experiments show that the muscle fiber-specific expression of Neuralized, an E3-ubiquitin ligase required in the Delta-Notch signal transduction process for Delta endocytosis (Skwarek et al., 2007), is significantly upregulated following muscle injury (Fig. 4R-T). Taken together, these findings imply that signaling between muscle fiber associated Delta ligand and satellite cell associated Notch receptor is required for the proliferative mitotic activity of satellite cells in response to muscle injury (Fig. 4U).

Discussion

The identification and characterization of satellite cells in *Drosophila* indicates that stem cell lineages act not only in the development of flight muscle as reported previously (Gunage et al., 2014), but also have a role in the mature muscle of the adult. Thus, as in vertebrates, the *Drosophila* satellite cells are lineal descendants of the muscle-specific stem cells generated during embryogenesis, become intimately associated with adult muscle fibers, and remain quiescent under normal circumstances, but become mitotically active and generate progeny that fuse with the injured

fibers following injury. The remarkable similarities in lineage, structure and function of satellite cells in flies and vertebrates imply that the role of these adult-specific muscle stem cells is evolutionarily conserved and, hence, are likely to manifest in other animals as well. Satellite cells have been identified in a crustacean (Parhyale hawaiensis) during limb regeneration (Konstantinides et al., 2014). Moreover, preliminary experiments in bees (Apis florea) indicate that adult muscle injury results in Notch-dependent proliferation of satellite cells as in *Drosophila* (Gunage, unpublished observation). It will now be interesting to determine if comparable satellite cells are also present in adult musculature of other key protostome and deuterostome invertebrate phyla such as molluscs, annelids and echinoderms.

In vertebrates, satellite cells can undergo symmetric divisions which expand the stem cell pool and asymmetric divisions in which they self-renew and also generate daughter cells that differentiate into the fusion-competent myoblasts required for muscle regeneration and repair (Abmayr and Pavlath, 2012; Brack and Rando, 2012). In *Drosophila*, symmetric and asymmetric division modes are seen during development in the muscle stem cell-like AMPs; Notch signaling controls the initial amplification of AMPs through symmetric divisions, the switch to asymmetric divisions is mediated by Wingless regulated Numb expression in the AMP lineage, and in both cases the wing imaginal disc acting as a niche provides critical ligands for these signaling events (Gunage et al., 2014). It will be important to determine if the fly satellite cells, as lineal descendants of AMPs, manifest similar cellular and molecular features in their proliferative response to muscle injury and, thus, recapitulate myogenic developmental mechanisms in the

regenerative response of adult muscle. It will also be important to investigate if the mature muscle acts as a niche in this process.

In recent years, *Drosophila* has proven to be a powerful genetic model system for unraveling the fundamental mechanisms of muscle development and stem cell biology, and in both respects many of the findings obtained in the fly have been important for the analysis of corresponding mechanisms in vertebrates (Roy and VijayRaghavan, 1999; Daczewska et al., 2010; Abmayr and Pavlath, 2014; Egger et al., 2008, 2010; Homem and Knoblich, 2012; Jiang and Reichert, 2014). With the identification of satellite cells in *Drosophila* the wealth of classical and molecular genetic tools available in this model system can now be applied to the mechanistic analysis adult-specific stem cell action in myogenic homeostasis and repair. Given the evidence for age and disease-related decline in satellite cell number and function in humans (e.g. Chang and Rudnicki, 2014), this type of analysis in *Drosophila* may provide useful information for insight into human muscle pathology.

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Author contributions.

The project was initiated and developed by R.D.G. and K.V. All the experiments were carried out by R.D.G. Data analysis and manuscript writing was done by R.D.G., H.R and K.V.

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Figure legends

Figure 1

Cells of the AMP lineage persist unfused in adult muscle.

(A) Schematic depicting possible differential fate of muscle stem cells versus postmitotic myoblasts. Myoblasts from wing imaginal disc (cells from layer 2 and 3, marked by blue boxed arrow) fuse to form multinucleated flight muscles. The fate of muscle stem cells (cells adjacent to epithelium, marked by green boxed arrow) is unknown. (B) Schematic depicting visualization of fused versus unfused AMP lineal descendants labeled with membrane-tethered GFP. Fusion of cells results in dilution of label in large multicellular muscle cell membrane. Label of unfused cells persists in intact membrane enclosing a single nucleus. (C) Simplified schematic depicting unfused muscle stem cells associated with flight muscles. (D, E) Flight muscles labeled with membrane-tethered GFP (green, anti-GFP immunolabeling), phalloidin (red; dotted lines mark muscle boundaries) and TOPRO-3 (blue). Single confocal optical section. GFP-labeled cells represent a MARCM clone (Dmef2 driving UAS mCD8::GFP) induced in third instar (~120 h AEL) and recovered in the adult stage. Associated with the phalloidin labeled muscle cells are unfused muscle stem cells labeled with GFP. Insets show enlarged views of unfused cells. n=12 (F, G) 3-D reconstruction of MARCM labeled cells and phalloidin labeled flight muscle from same preparation as in D, E. Confocal section stack. Unfused cells (green, anti-GFP immunolabling) are present throughout the length of muscle and are anatomically intercalated between the muscle fibers (red, phalloidin). (H) Optical orthogonal section of the same preparation as in C, D showing MARCM labeled unfused AMP lineal descendants associated with

muscle fibers (cells labeled as in D, E). Single confocal optical section. (I) Schematic of G, H. Scale bars 50μm

Figure 2

Unfused muscle-associated cells have ultrastuctural features of satellite cells.

(A) Transmission electron micrographs of the adult flight muscle. Myonuclei are large round structures surrounded by double membranes; outlined with white dotted lines. The cytoplasm of the muscle syncytium shows distinct sarcomeres (marked as S), nucleus (marked as N) and mitochondria (marked as M).

(B) Example of mononucleated unfused cells. The intact cell membrane (marked by a green double-headed arrow) of these cells can be distinctly seen in close apposition to muscle membrane (marked by a long red arrow) and adjacent to the basement membrane (marked by a yellow arrow) of the muscle fiber. These unfused cells contain few cytoplasmic organelles and have a wedged shaped nucleus (marked by white dotted line). n= 8. Scale bar 1μm.

Figure 3

Muscle injury results in proliferative activity of satellite cells.

(A) Thoracic flight muscles showing the site of an injury indicated by dotted circle (white). Whole mount of flight muscles labeled by phalloidin (green) with all nuclei are labeled by TOPRO-3 (blue). The injury was induced with a tungsten needle in to the flight muscles at position shown in Fig. 1 C (Marked by asterisk). n= 10 (B-D) Immunolabeling of mitotically active cells in flight muscles labeled for PH-3 (red, anti-phopsphohistone-3 immunolableling), phalloidin (green) and TOPRO-3 (blue) in an uninjured control (B) and an injured muscle

(C). Multiple optical sections. A marked increase in mitotically active cells (red arrows) is seen in injured muscles as compared to controls. Mitotically inactive unfused cells are indicated by white arrows. (D) Quantification of the number of PH-3 labeled cells in control versus injured muscle. n=15. (E-H) Injured flight muscles showing GFP-positive MARCM labeled AMP lineages (clones induced as described in figure 1), co-immunolabeled for PH-3. Clonally derived AMP lineage cells (green, anti-GFP immunolabeling; marked by red arrows) show co-localisation with PH-3 expression (red, anti-PH-3 immunolabeling) in TOPRO-labeled background (blue). n= 8 (I-N) Fusion of a subset of AMP lineal cells labeled by nls-GFP (nuclear localizing GFP) with injured muscle fibers. Multiple sections shown in I; single orthogonal optical sections shown in J-N. MARCM clonal labeling with nls-GFP (Dmef2-Gal4 driving UAS-nls-GFP) was induced in the adult prior to injury and recovered one day later. Clonal analysis shows presence of GFP labeled nuclei of AMP lineal cells in the injured muscle (red, anti-MHC immunolabeling). In N, an unfused GFP labeled cell can also be seen indicated by white dotted circle. (O) Simplified schematic of fused versus unfused AMP lineal cells in injured muscle. n= 8, Scale bar 50μm. In this and all subsequent quantification results, data is presented as mean ± standard error (Student's t test); p-values < 0.01, **; p-values < 0.001, ***.

Figure 4

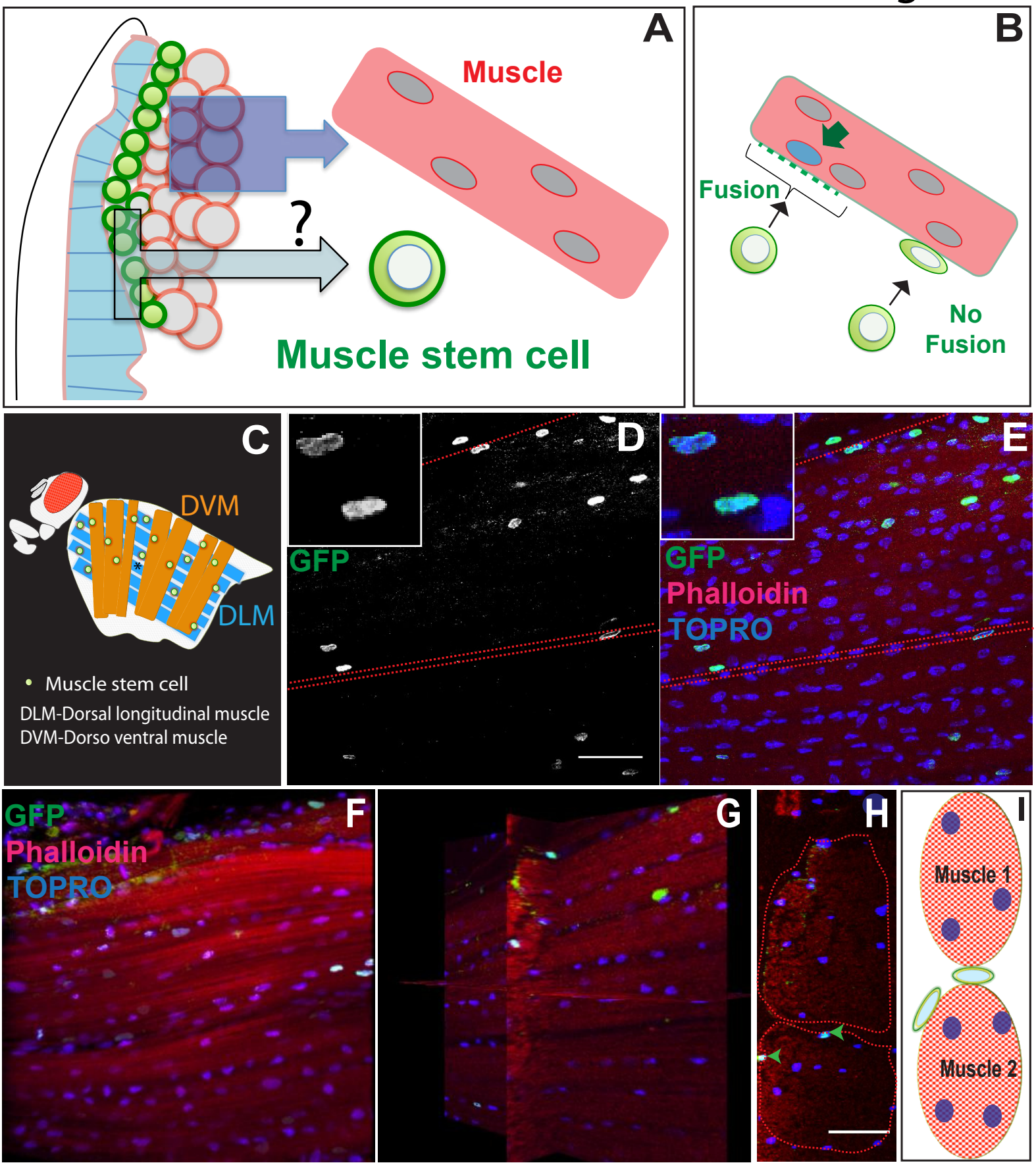
Activation of satellite cell proliferation is controlled by Notch signaling.

(A–C) Notch expression in uninjured flight muscles. Optical section through muscle showing a MARCM induced labeling of a satellite cell (green; mCD::GFP immunolabeled) manifesting high Notch expression (red, anti-Notch intracellular domain immunolabeling) in a TOPRO

labeled (blue) background. MARCM clones induced as described in figure 1. n=6 Scale bar, 10 μm. (D) Quantification of the number of PH-3 labeled cells in injured muscle of Notch temperature sensitive allele flies at restrictive (29^0C) versus permissive (17^0C) temperature. n= 12 (E, F) Multiple optical sections of flight muscles stained for mitotically active cells with PH-3 labeling (red, anti-PH-3 immunolabeling) in a myosin heavy chain (green, anti-MHC) and TOPRO3 (blue) labeled background in control flies (E) versus flies in which Notch is downregulated by Dmef2-Gal4, TubGal80ts > UAS Notch RNAi (F). (In this experiment, Gal80 repression was relieved post eclosion by shifting cultures from 18°C to 29°C.) (G) Quantification of mitotically active PH3 positive satellite cells in control versus Notch downregulated flies. n= 12 (H-N) Optical orthogonal section of flight muscle stained for NRE-GFP (Notch responsive element promoter fusion of E(Spl) driving expression of GFP), a reporter for canonical Notch signaling. In injured muscle (K-N), activation of NRE-GFP (green, anti-GFP) can be visualized in satellite cells (marked in dotted red circles); this is not seen in uninjured controls (H-J). (N) Quantification of the number of NRE-GFP labeled cells in uninjured versus injured muscles. n= 8 (O, P) Optical section of flight muscles stained for PH-3 (green, anti-PH3 immunolabeling) and Delta (red, anti-Delta immunolableing) expression in a TOPRO3 (blue) background in control flies (O) versus flies in which Delta is downregulated by Act88F-Gal4, TubGal80ts > UAS Delta RNAi (P). (In this experiment, Gal80 repression was relieved post eclosion by shifting cultures from 18°C to 29°C.) Delta downregulation decreases the number of mitotically active PH-3 expressing cells in comparison to control (Q). Quantification of the number of PH-3 expressing cells in control versus Delta downregulated flies; Delta downregulation is achieved by targeted Delta-RNAi knockdown as well as by targeted

dominant negative Delta (DN Delta) expression. n= 9 (R, S) Flight muscles stained for Neuralised (Neur) (red, anti-Neur immunolabeling) and co-labeled by phalloidin (green). Multiple optical sections. In comparison to controls (R), injured muscles show elevated Neur (S). (T) Quantitation of Neur expression (in arbitrary units of immunofluorescence) in control versus injured muscle. n= 7. Scale bar, 50 μm. (U) Schematic of model in which signaling between a muscle fiber associated Delta ligand and a satellite cell associated Notch receptor is required for mitotic activity of satellite cells in injured muscle.

Figure 1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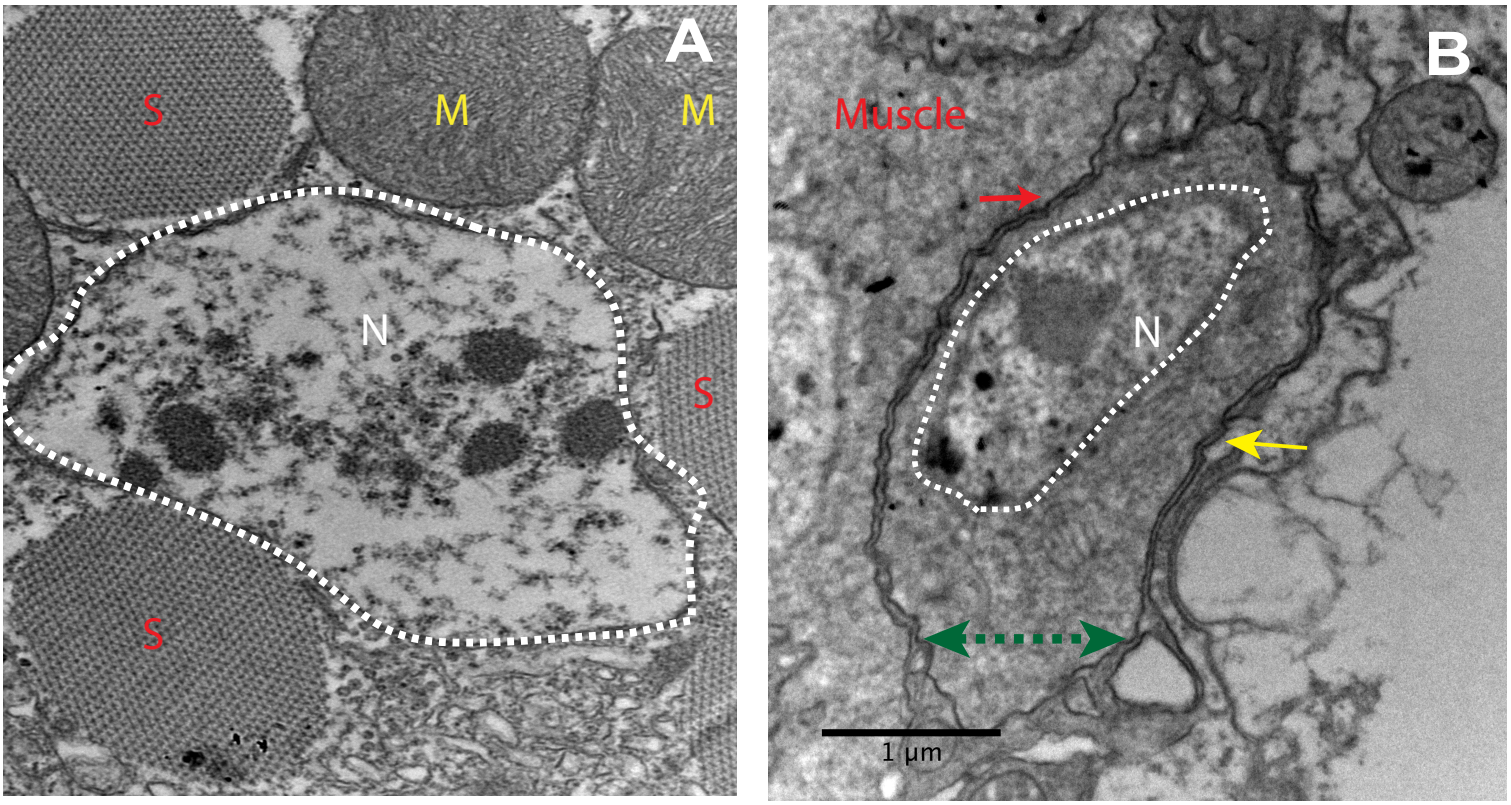
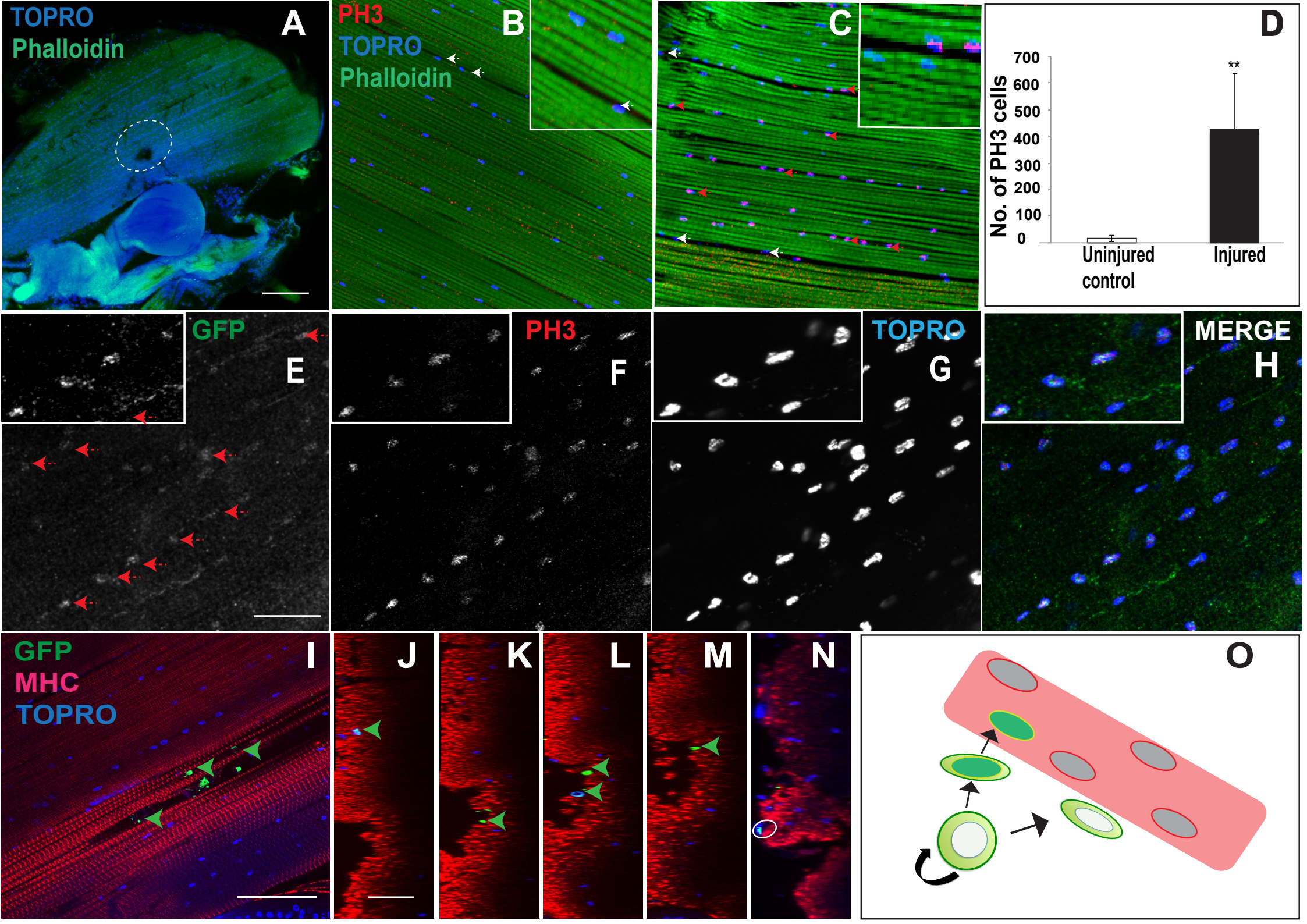
?
Muscle stem cell
B
Fusion
No
Fusion
C
DVM
DLM
Muscle stem cell
DLM-Dorsal longitudinal muscle
DVM-Dorso ventral muscle
D
GFP
E
GFP
Phalloidin
TOPRO
F
GFP
Phalloidin
TOPRO
G
H
I
Muscle 1
Muscle 2

Figure 2




TOPRO
Phalloidin
A
PH3
TOPRO
Phalloidin
B
C
D
No. of PH3 cells
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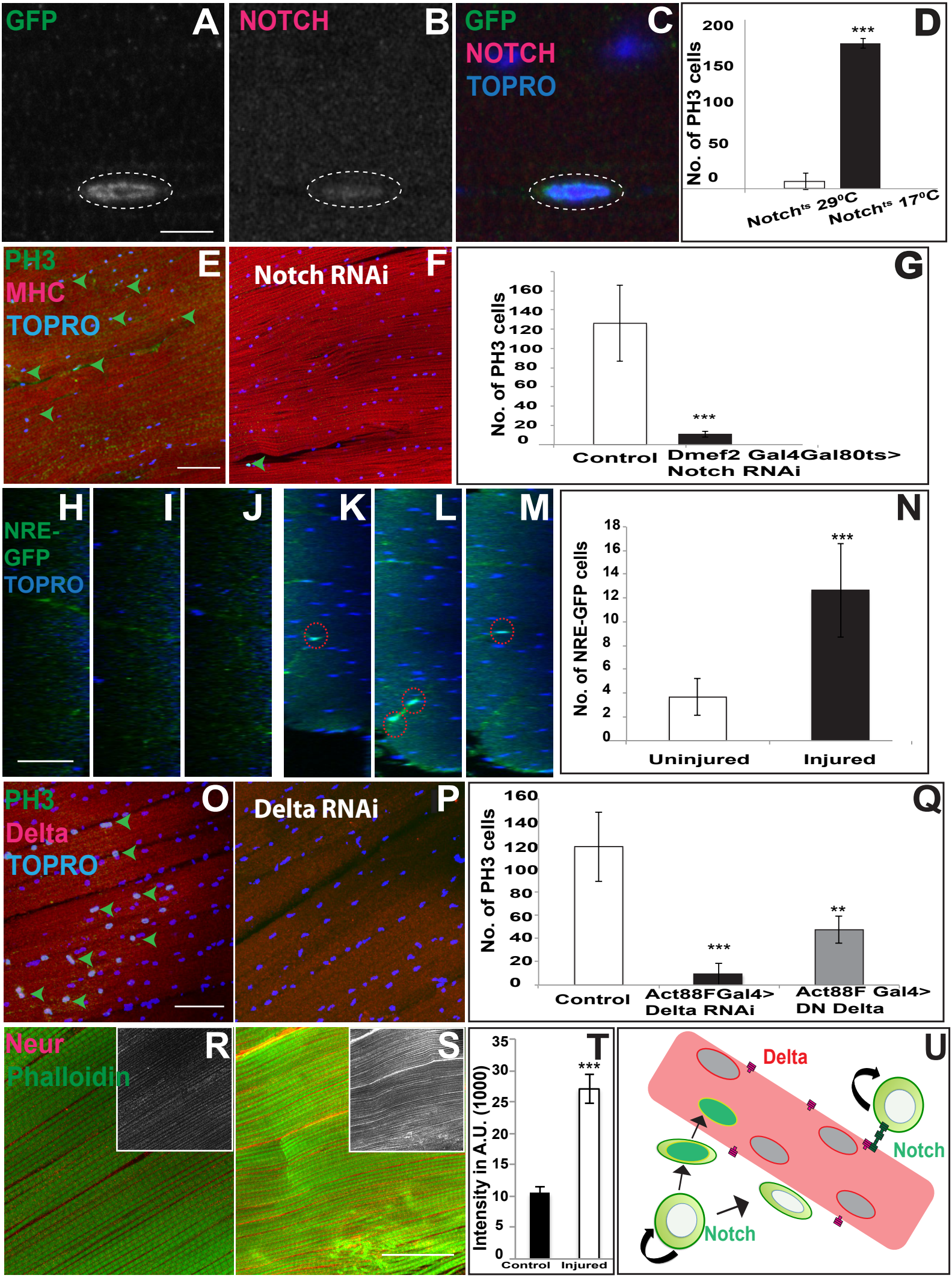
600
500
400
300
200
100
0
**
Uninjured control
Injured
GFP
E
PH3
F
TOPRO
G
MERGE
H
GFP
MHC
TOPRO
I
J
K
L
M
N
O

Figure 4


GFP
A
NOTCH
B
GFP
NOTCH
TOPRO
C
D
No. of PH3 cells
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150
100
50
0
***
Notch^ts 29°C
Notch^ts 17°C
PH3
MHC
TOPRO
E
Notch RNAi
F
G
No. of PH3 cells
160
140
120
100
80
60
40
20
0
***
Control
Dmef2 Gal4Gal80ts>
Notch RNAi
H
I
J
K
L
M
NRE-
GFP
TOPRO
N
No. of NRE-GFP cells
18
16
14
12
10
8
6
4
2
0
***
Uninjured
Injured
PH3
Delta
TOPRO
O
Delta RNAi
P
Q
No. of PH3 cells
160
140
120
100
80
60
40
20
0
***
**
Control
Act88FGal4>
Delta RNAi
Act88F Gal4>
DN Delta
Neur
Phalloidin
R
S
T
Intensity in A.U. (1000)
35
30
25
20
15
10
5
0
***
Control
Injured
U
Delta
Notch
Notch