Adult Muscle Formation Requires *Drosophila* Moleskin for Proliferation of Wing Disc-Associated Muscle Precursors

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ABSTRACT Adult muscle precursor (AMP) cells located in the notum of the larval wing disc undergo rapid amplification and eventual fusion to generate the *Drosophila melanogaster* indirect flight muscles (IFMs). Here we find that loss of Moleskin (Msk) function in these wing disc-associated myoblasts reduces the overall AMP pool size, resulting in the absence of IFM formation. This myoblast loss is due to a decrease in the AMP proliferative capacity and is independent of cell death. In contrast, disruption of Msk during pupal myoblast proliferation does not alter the AMP number, suggesting that Msk is specifically required for larval AMP proliferation. It has been previously shown that Wingless (Wg) signaling maintains expression of the Vestigial (Vg) transcription factor in proliferating myoblasts. However, other factors that influence Wg-mediated myoblast proliferation are largely unknown. Here we examine the interactions between Msk and the Wg pathway in regulation of the AMP pool size. We find that a myoblast-specific reduction of Msk results in the absence of Vg expression and a complete loss of the Wg pathway readout β -catenin/Armado (Arm). Moreover, *msk* RNA interference knockdown abolishes expression of the Wg target Ladybird (Lbe) in leg disc myoblasts. Collectively, our results provide strong evidence that Msk acts through the Wg signaling pathway to control myoblast pool size and muscle formation by regulating Arm stability or nuclear transport.

KEYWORDS *Drosophila melanogaster*; indirect flight muscles; Moleskin; proliferation

STEM cell pool proliferation is critical in the regulation of tissue size and organization in normal development and mediates repair processes following injury (Micchelli and Perrimon 2006; Gonzalez 2007; Egger *et al.* 2008; Jiang and Edgar 2012). The extent of cell proliferation required to generate different tissues is variable and generally influenced by the size of an initial precursor pool, balanced by the frequency of cell division and/or subsequent cell differentiation. For example, a lack of neural stem proliferation during neural circuit formation can result in microcephaly in mice (Homem *et al.* 2015). Studies performed in both mice and *Drosophila* show that intestinal stem cell proliferation dictates tissue maintenance and repair (Jiang and Edgar 2012). Coordination between cell proliferation and cell differentiation is

critical for the formation and maintenance of larval blood cell generation and ovarian development in *Drosophila* (Gilboa 2015). Ultimately, common mechanisms unite proliferative processes that form diverse tissues.

A number of evolutionarily conserved signaling pathways are known to regulate stem cell proliferation. For example, Wingless (Wg)/Wnt signaling is the principle regulator of mammalian intestinal stem cell proliferation (Jiang and Edgar 2012). Similarly, Hippo signaling maintains lung cell homeostasis by controlling the proliferation of epithelial stem cells (Lange *et al.* 2015). Although some factors that regulate stem cell proliferation have been widely studied (Brack *et al.* 2008; Takashima *et al.* 2008; Benmimoun *et al.* 2012; Chen *et al.* 2012; Shim *et al.* 2013), additional components and detailed mechanisms controlling stem cell proliferation are not clearly understood.

The indirect flight muscles (IFMs) of *Drosophila melanogaster* serve as a good model system to understand mechanisms that regulate stem cell proliferation (Fernandes *et al.* 1991; Roy and VijayRaghavan 1999; Dutta and VijayRaghavan 2006). The IFMs are subdivided into two groups: the dorsoventral muscles

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and the dorsal longitudinal muscles (DLMs). In DLM formation, muscle stem cells called adult muscle precursors (AMPs) are set aside in embryogenesis and will eventually give rise to this set of IFMs (Bate *et al.* 1991; Figeac *et al.* 2007, 2011). The AMPs remain in an undifferentiated state and undergo rapid rounds of proliferation in the notum region of the wing disc during the second (L2) and third (L3) larval instar stages to generate ~2500 myoblasts within a period of 120 hr (Gunage *et al.* 2014). At the onset of pupation, most thoracic larval muscle fibers undergo histolysis. However, three dorsal oblique muscles are spared and split into six fibers that serve as DLM templates, also called organizer or founder muscles (Roy and VijayRaghavan 1998; Bernard *et al.* 2003). The AMPs undergo an additional round of proliferation and myoblast fusion to form the eventual six DLM fibers that are approximately one-third of their final size. These muscles increase in volume for the remaining 3 days of pupal development and each DLM ends up with ~3000 myonuclei (Kopec 1923). Thus, the rapid proliferation of muscle stem cells during larval and pupal development is critical for proper IFM formation.

A network of transcription factors are responsible for the massive proliferative increase in wing disc-associated AMPs. Twist (Twi) and Notch are required for maintaining myoblasts in a proliferative phase to block muscle differentiation (Bate *et al.* 1991; Anant *et al.* 1998; Bernard *et al.* 2010). Disrupting Notch function in the AMPs downregulates Twi expression and results in premature differentiation (Anant *et al.* 1998). In contrast, Mef2 is the major differentiation factor that promotes IFM formation and is activated by the antidifferentiation protein Twi (Ranganayakulu *et al.* 1995; Cripps and Olson 1998; Bryantsev *et al.* 2012). This paradox is resolved by findings that Twi and Notch activate the Holes in muscle (Him) transcription factor to prevent premature muscle differentiation through suppression of Mef2 activity (Liotta *et al.* 2007; Soler and Taylor 2009).

The transcriptional activity required for the large increase in the larval AMP pool size is developmentally regulated. Notch pathway activation is initiated in embryogenesis and extends into larval development to promote AMP amplification, while soluble Wg protein released from the disc epithelial cells in L3 larvae supersedes Notch signaling (Gunage *et al.* 2014). Wg then acts through Arm and Pangolin/T-cell factor (TCF) upstream of the transcription factor Vestigial (Vg) to control the myoblast precursor pool and subsequent IFM formation (Sudarsan *et al.* 2001). In support of this, expression of dominant-negative TCF in myoblasts decreases Vg protein expression, reduces the AMP pool size, and impairs IFM formation (Sudarsan *et al.* 2001). The wing disc-associated myoblasts that express Vg and low levels of another transcription factor called Cut generate the IFMs, while myoblasts that express high levels of Cut give rise to direct flight muscles (DFMs). Thus, Vg and Cut act in a mutually repressive manner during muscle formation to generate distinct muscle types.

How the Wg and Notch signaling pathways integrate with Vg and other wing disc-associated proteins in the amplification of AMPs is still unclear. Here we demonstrate a new role for

Drosophila Moleskin (Msk)/Importin-7 (Dim7) in regulation of the adult myoblast pool size. Msk is a member of the Importin- β superfamily of proteins and is involved in nuclear protein transport in both vertebrates and invertebrates (Görlich *et al.* 1997; Mason and Goldfarb 2009). Studies in vertebrate skeletal myogenesis demonstrate that Importin-7 is required for the nucleocytoplasmic shuttling of extracellular signal-related kinase (ERK) to regulate myoblast proliferation and differentiation (Michailovici *et al.* 2014). Similarly, *Drosophila* Importin-7 regulates the nuclear transport of ERK and in turn influences cell proliferation in wing disc development (Marenda *et al.* 2006). However, Msk also has functions independent of its nuclear shuttling role during embryonic myogenesis. An Elmo-Msk protein complex localizes to the sites of myotendinous junction formation and *msk* mutants exhibit muscle detachment phenotypes (Liu and Geisbrecht 2011; Liu *et al.* 2013).

In this article, we highlight a new role for Msk in the regulation of muscle stem cell numbers during *Drosophila* adult muscle formation. We find that blocking Msk function in the wing disc-associated muscle precursors results in a drastic reduction in the overall size of the AMP pool and leads to the absence of DLMs. This lack of muscle formation is a result of impaired larval AMP amplification, as disruption of Msk function in pupal myoblast proliferation does not affect the myoblast pool size and has a minor effect on DLM formation.

Materials and Methods

Fly strains and genetics

All fly lines used in this study were grown on standard cornmeal medium at 25° unless otherwise stated. The following fly strains were obtained from published sources: *da-Gal4* [for quantitative PCR (qPCR); from M. Dushay], *1151-Gal4* (myoblast-specific driver from L. S. Shashidhara), and *rp298-Gal4* (founder cell-specific driver from S. Abmayr). The following fly strains were obtained from the Bloomington stock center: upstream activator sequence (UAS)-nuclear localization signal (nls)-*GFP* [*w*[1118]; *P*{*w*[+mC]=UAS-*GFP.nls*}14 (BL4775)]; UAS-*GFP* RNA interference (RNAi) [*w*[1118]; *P*{*w*[+mC]=UAS-*GFP.dsRNA.R*}143 (BL9331)]; UAS-DN-TCF [*y*[1] *w*[1118]; *P*{*w*[+mC]=UAS-*pan.dTCFDeltaN*}4 (BL4784)], UAS-*armS10* [*P*{UAS-*arm.S10*}C, *y*[1]*w*[1118]] (BL4782)]; UAS-*msk* full length (FL) [*w*[*]; *P*{*w*[+mC]=UAS-*msk.L*}47M1/CyO (BL23944)]; UAS-*sgg* [wild type (WT)] [*w*[1118]; *P*{*w*[+mC]=UAS-*sgg.B*}MB5 (BL5361)]; UAS-*sgg*(Y241F) [*w*[1118]; *P*{*w*[+mC]=UAS-*sgg.Y241F*}2 (BL6817)]; and two UAS-*msk* RNAi lines [*y*[1] *v*[1]; *P*{*y*[+t7.7] *v*[+t1.8]=TRiP.JF02727}attP2 (BL-27572)] and [*y*[1] *v*[1]; *P*{*y*[+t7.7] *v*[+t1.8]=TRiP.HMS00020}attP2 (BL33626)] (Liu *et al.* 2013). The following fly stocks were generated in the laboratory using standard genetic crosses: *1151-Gal4*; UAS-*Gal80^{ts}* (for temporal regulation of transgene expression); UAS-DN-TCF; UAS-*msk* FL (for rescue of DN-TCF); UAS-*GFP*, UAS-*msk* RNAi (control for *armS10* rescue); UAS-*armS10*, UAS-*msk* RNAi (for rescue of *armS10*); UAS-*msk* FL; UAS-*sgg* (WT) [for rescue of shaggy (*sgg*)]; UAS-*msk* FL; and UAS-*sgg* (Y241F) (for rescue of activated *sgg*).

Immunostaining

Appropriately staged wandering L3 larvae were selected to analyze the role of Msk in regulating muscle stem cell number in wing and leg imaginal discs. Wing discs from individual larvae were dissected and fixed in 4% formaldehyde for 25 min at room temperature followed by immunohistochemistry. To examine the effect of Msk function in adult IFM muscle formation, 0-hr pupae (white pupae) were collected and aged to specific time points: 20 hr after puparium formation (APF) (splitting of larval template/organizer cell is completed) or 24 hr APF (initial steps of DLM patterning are completed). Pupal preparations were dissected, fixed, and immunostained. The myoblast pool in both wing and leg discs were labeled using rabbit anti-Twi (1:300; K. Jagla), mouse anti-Ladybird (Lbe) (1:1000; K. Jagla), guinea pig anti-Earthbound 1 (anti-Ebd) (1:4000; Y. Ahmed), mouse anti-Cut [1:100; Developmental Studies Hybridoma Bank (DSHB)], rabbit anti-Vg (1:100; S. Carroll), and rabbit anti-Mef2 (1:100; B. Patterson). Cell death was analyzed using Acridine Orange (AO) staining (Sigma Chemical, St. Louis, MO) and TUNEL assays (Roche) using standard protocols (Milán *et al.* 1997). Muscle differentiation was examined using mouse anti-Myosin heavy chain (anti-MHC) (1:500; S. Abmayr). Myoblast proliferation was monitored using rabbit anti-phospho-Histone 3 (anti-PH3) (1:100; Millipore, Bedford, MA). Mouse anti- β -Catenin/Arm (1:100; DSHB) was analyzed as a readout of Wg signaling. Myonuclei were labeled using rabbit anti-Erect wing (anti-Ewg) (1:2000; Y. Ahmed) and developing fibers were monitored using mouse anti-22C10 (1:100; DSHB). Secondary antibodies for fluorescent immunostaining were Alexa Fluor 488 and Alexa 546. Immunostained preparations were imaged on a Carl Zeiss (Thornwood, NY) 700 confocal microscope and images were processed using Carl Zeiss ZEN software and Photoshop Elements 12.

qPCR to verify Msk knockdown by RNAi

Three samples of five larvae each were prepared for the *da-Gal4/+* and *da-Gal4 > msk RNAi* genotypes. Many *da-Gal4 > msk RNAi* larvae died before reaching the L3 stage, so five L2 larvae were homogenized in the buffer provided in the kit. Three RNA samples for each phenotype were used for analysis. RNA was generated using the RNeasy Mini Kit (QIAGEN, Valencia, CA). After elution, RNA concentrations were determined using Thermo Scientific's NanoDrop 1000. Single strand complementary DNA (cDNA) was generated from 150-ng RNA using the SuperScript III First-Strand Synthesis System Kit (Invitrogen, Carlsbad, CA). For the qPCR reactions, each cDNA solution was diluted to 1:50 and mixed with SYBR Select Master Mix for CFX (Applied Biosystems, Foster City, CA). *rp49* was used as the reference gene. Primers for the qPCR reactions were synthesized by Integrated DNA Technologies:

msk: left, 5'-TTGCGCGCAACTATTGATCC-3'; right, 5'-CTTGAGGTAGACAGCACCGG-3'.
rp49: left, 5'-GCCCAAGGGTATCGACAACA-3', right, 5'-GCGCTTGTTTCGATCCGTAAC-3'.

Reactions were run in triplicate using the Bio-Rad (Hercules, CA) CFX96 Touch Real-Time PCR Detection System with

CFX Manager Software. The average of the triplicates was used to calculate the $2^{-\Delta\Delta Ct}$ values (normalized fold expression).

Quantitation and statistical analysis

The size of the myoblast pool was quantitated using two different methods: (1) Myoblast density was determined by counting the total number of Twi(+), Mef2(+), or Ebd(+) nuclei in three random regions (1600 μm^2 area each) of the wing imaginal disc-associated myoblasts; and/or (2) by counting the total number of wing disc-associated Twi(+) myoblasts in single nodal planes of 1- μm thickness. Myoblast proliferation was calculated by determining the percentage of PH3(+)/Cut(+) notum myoblasts. Wg signaling was monitored by comparing the fraction of Twi(+) or Mef2(+) myoblasts that colocalized with Arm nuclei in a single nodal plane. $N \geq 15$ individuals for each genotype in each experiment. Fiber formation in the pupal stages was analyzed by comparing the total number of 22C10(+) developing fibers per hemi-segment between the control and experimental animals at specific time points. IFM myonuclei were monitored by counting the total number of Ewg(+) nuclei per fiber (Mukherjee *et al.* 2011). $N = 6-8$ individuals for each genotype were quantified.

Myoblast quantifications were performed using the "Analyze Particles" function in ImageJ, recorded in an Excel spreadsheet, and imported into Graphpad Prism 6.0 software for the generation of graphs and statistical analysis. The column statistics function was used to confirm statistically significant sample sizes and normality. All error bars represent the mean $\pm$ SEM. Statistical significances were determined using either Student's *t*-tests, Mann-Whitney tests, or one-way ANOVA followed by a Bonferroni *post hoc* analysis. Differences were considered significant if $P < 0.05$ and are indicated in each figure legend.

Data availability

The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. All reagents used in this study are available upon request.

Results

Msk is required in the larval AMPs for IFM formation

Our laboratory previously found that *msk* is essential for the establishment of muscle-tendon attachment in *Drosophila* embryogenesis (Liu and Geisbrecht 2011; Liu *et al.* 2013). As an extension of this work, we sought to evaluate the contribution of Msk in adult myogenesis. We and others have found that *msk* mutants are lethal prior to pupal stages, thus precluding analysis of adult IFM formation (Lorenzen *et al.* 2001; Liu and Geisbrecht 2011; Liu *et al.* 2013; Natalizio and Matera 2013). To circumvent this limitation and to examine the role of Msk in the pupal stages of IFM development, we used the binary Gal4/UAS expression system to knockdown Msk using RNAi. First, we confirmed that two independent

UAS-*msk* RNAi lines effectively reduced *msk* transcript levels using qPCR (Supplemental Material, Figure S1 in File S1). The stronger UAS-*msk* RNAi^{HMS} is used for the remainder of our studies. Knockdown of Msk was further confirmed in this line using newly generated antisera against the Msk protein (Figure S1 in File S1).

To examine whether a reduction in Msk affects the pool of actively dividing myoblasts that give rise to the IFMs, knockdown of Msk was accomplished using *1151-Gal4*. Analysis of GFP fused to the SV40 nuclear localization signal (UAS-*nls-GFP*) confirms expression of the *1151-Gal4* driver in proliferating adult myoblasts located in the notum region of the L3 wing disc (Figure 1, A and B; yellow box) (Fernandes *et al.* 2005). Control discs of *1151-Gal4* alone possess ~2500 myoblasts by the end of the L3 stage and can be visualized using the myoblast markers *Tw* (Figure 1, C and F) or *Mef2* (Figure 1I). Knockdown of *msk* transcript in these same proliferating myoblasts substantially reduced the myoblast pool (Figure 1, G and J). While the position of the remaining myoblasts within the notum varied, the reduction in the overall myoblast pool size remained constant among different samples (Figure 1, H and K). Note that basal expression of the UAS-*msk* RNAi line with no driver (Figure S1 in File S1) or knockdown of GFP as a negative control (*1151 > GFP* RNAi) (Figure 1, D and E) did not alter the number of myoblasts.

The *msk* RNAi-induced reduction in myoblast density suggests that Msk may be required for cell death, muscle differentiation, and/or cell proliferation in the AMPs. To examine if apoptosis could be a cause of the decreased myoblast pool, we examined *1151-Gal4* control and *1151 > msk* RNAi notum myoblasts for activated Caspase 3 or AO positive cells. We failed to detect any indication of cell death in the AMPs (Figure S2 in File S1). Next we immunostained L3 wing discs with an antibody against MHC which detects differentiated muscle tissue (Lovato *et al.* 2005). No staining was observed in control or *msk* RNAi myoblasts (Figure S3 in File S1), indicating that premature muscle differentiation was not a cause of AMP reduction. The marker PH3 is specific for cells undergoing mitosis. On average, a small fraction of Cut-labeled myoblasts also stain for PH3 (Figure 1, L and N). A decrease in Msk reduced the fraction of PH3(+)/Cut(+) myoblasts (Figure 1, M and N). Thus, here we conclude that Msk is required for myoblast proliferation in the notum region of L3 wing discs.

The large increase in proliferating myoblasts during the larval stages ensures a sizable myoblast pool during fusion to generate the adult IFMs. By 12 hr APF, larval muscle histolysis is complete and myoblasts migrate toward three persistent DLM muscle templates for fusion and muscle growth. Rapid myoblast fusion continues between 12 and 18 hr APF and induces splitting of the three larval scaffolds into six DLM fibers (Weitkunat and Schnorrrer 2014). Myoblast nuclei labeled with anti-Ewg antisera are present in the developing DLMs marked with *1151*-driven GFP expression at 20 hr APF (Figure 2A) and 24 hr APF (Figure 2F). These data are consistent with previous reports where *1151* is observed in the

developing IFM fibers in pupal development (Anant *et al.* 1998; Dutta *et al.* 2004). This continued expression of the *1151* promoter in proliferating and fusing myoblasts allowed us to test the effect of persistent *msk* RNAi knockdown during pupal DLM development. Control preparations of *1151-Gal4* alone or *1151 > GFP* RNAi both showed the full complement of six IFM fibers at 20 hr APF (Figure 2, B, C, and E; *) and 24 hr APF (Figure 2, G, H, and J; *). Reduction of *msk* during proliferation of the larval AMPs dramatically decreased the number of muscle fibers analyzed at both 20 hr (Figure 2, D and E) and 24 hr APF (Figure 2, I and J). Notably, the remaining muscles appear to be larval templates as the Ewg-stained nuclei are larger than the nuclei present in developing DLM fibers. Together, these results suggest that Msk is required to produce a minimal number of myoblasts sufficient for fiber size and/or splitting. Failure to maintain this proliferative state may cause muscle degeneration since we never observed three larval templates in *1151*-driven *msk* RNAi individuals.

To further determine if Msk is required in all myoblasts or if it specifically affects a subset of myoblasts, *msk* RNAi was expressed under control of the *duf/kirre* promoter (*rp298-Gal4*). *rp298* is expressed in the three larval templates that serve as founder muscles in organizing future DLM fiber development during myoblast fusion (Fernandes *et al.* 1991; Dutta *et al.* 2004). A subset of wing disc-associated myoblasts express GFP under control of the *rp298* promoter (Figure 3A). Compared to *rp298-Gal4* (Figure 3B) or *rp298 > GFP* RNAi (Figure 3C) controls, fewer myoblasts were observed after a reduction in *msk* RNAi levels (Figure 3D). Accordingly, this reduction in myoblast number (Figure 3E) also resulted in decreased fiber number. The six DLMs normally present at 20 hr APF (Figure 3, F–H; *) or 24 hr APF (Figure 3, K–M; *) were decreased to ~4 fibers upon induction of *msk* RNAi by *rp298-Gal4* (Figure 3, I and J; *) at 24 hr APF (Figure 3, N and O; asterisks). These data implicate Msk as an important player in the generation and/or maintenance of the myoblast pool, both in founder cells and fusing myoblasts.

Our results thus far show that a reduction in Msk activity was initiated during larval AMP proliferation and persisted through pupal myoblast proliferation, myoblast fusion, and fiber splitting. While the final number of DLM fibers was decreased upon *msk* RNAi by 24 hr APF (Figure 2J and Figure 3O), we could not distinguish between a requirement for Msk in generation of the larval or pupal myoblast pool. Therefore, we used the Gal4/Gal80^{ts} TARGET system to bypass the requirement for Msk during larval development and determine if Msk is essential for pupal myoblast proliferation (Brand and Perrimon 1993; Sudarsan *et al.* 2001; McGuire *et al.* 2004). Gal80^{ts} is a temperature-sensitive (ts) mutation that binds to and inactivates the Gal4 protein at permissive temperatures (18°). This inactivation prevents the Gal4 expression of UAS-driven elements. At restrictive temperatures (29°), the Gal80^{ts} protein loses its ability to repress Gal4 and allows for the induction of UAS transgenes. *1151-Gal4*; UAS-Gal80^{ts} control pupae at 24 hr APF

Figure 1 Msk is required for the generation of the wing disc-associated myoblast pool. (A and B) The *1151-Gal4* driver is used to express nls-GFP in all larval wing disc-associated myoblasts at the L3 stage. (A) Low magnification of the wing disc (white dotted outline). The yellow boxed region shows the location of the larval myoblasts in the notum (B). (C, D, F, G, I, and J) Maximum projection confocal microscopy images of the AMP pool in (C, D, F, and I) control or (G and J) *1151 > msk RNAi* L3 wing discs labeled with the myoblast markers (C, D, F, and G) Twi or (I and J) Mef2. Note that the myoblast pool (dotted line) is reduced upon disruption of (G and J) Msk compared to (C, D, and F) controls. (E, H, and K) Quantitation of myoblast density (per regions 1600 μm^2) in control (*1151-Gal4* or *1151 > GFP RNAi*) and *msk RNAi* (*1151 > msk RNAi*) wing discs labeled with (E and H) Twi or (K) Mef2. (L–N) PH3 staining to monitor proliferating notum myoblasts. More Cut(+) myoblasts also stain for PH3 in (L) control compared to (M) *msk RNAi* discs. (N) Bar graph showing the fraction of PH3(+)/Cut(+) myoblasts. Mean $\pm$ SEM. n.s., not significant. **** $P < 0.001$, *** $P < 0.005$. Bar, 50 μm .

possessed six DLM fibers (Figure 4, B and C; *). Individuals expressing *msk RNAi* were shifted to the nonpermissive temperature of 29° at 0 hr APF, which corresponds to the beginning of pupal development (Figure 4A). Dissection at 24 hr APF yielded a small, yet significant, reduction in fiber number

after a decrease in Msk function (Figure 4, F and G; *). This reduction is likely caused by a delay in fiber splitting (Figure 4G; $\leftarrow$) and not due to a lack of muscle formation from defective pupal myoblast proliferation or aberrant fusion, as the number of myoblasts observed in control or *msk RNAi*

Figure 2 Abrogated Msk function during larval myoblast proliferation reduces DLM fiber number. (A–D and F–I) Maximum projection confocal micrographs of DLM fibers at (A–D) 20 hr APF or (F–I) 24 hr APF. (A and F) Pupal myoblasts labeled with Ewg (red) are being incorporated into the developing DLM fibers (*) marked by 1151-driven GFP (green) at (A) 20 hr APF or (F) 24 hr APF through reiterative myoblast fusion events. (B–D and G–I) Developing DLMs are stained with 22C10 (green) to mark muscle fibers and Ewg (red) to label myoblasts. (B and G) 1151-Gal4 or (C and H) 1151 > GFP RNAi control animals have six DLM fibers (*) at (B and C) 20 hr APF or (G and H) 24 hr APF. (D and I) Little fiber formation is seen in 1151 > msk RNAi animals. (E and J) Quantitation of fiber number shows there are significantly fewer DLM fibers upon knockdown with *msk RNAi* animals compared to controls. Mean $\pm$ SEM. n.s., not significant. **** $P < 0.001$. Bar, 50 μ m.

knockdown muscle fibers were similar (Figure 4, D, E, H, and I).

Msk influences Wg signaling

IFM formation requires Vg, a transcriptional activator that is expressed at higher levels in the proximal myoblasts of the wing disc (Figure 5, A and B; yellow *) (Sudarsan *et al.* 2001; Bernard *et al.* 2003). In contrast, the Cut transcription factor is normally highest in myoblasts closest to the wing hinge (Figure 5, A and C; white $\leftarrow$) and gives rise to the DFMs. Vg staining is absent from the entire notum myoblast pool upon *msk RNAi* knockdown (Figure 5, D and E), while Cut protein persists in the remaining myoblasts (Figure 5, D and F). Thus, loss of Msk affects the presence of Vg, a key regulator of IFM development. Since Vg represses Notch in developing DLMs (Bernard *et al.* 2006), we examined whether loss of Vg via *msk RNAi* altered Notch signaling. However, the levels or subcellular localization of the Notch intracellular domain were not altered in control or *msk RNAi* wing discs (Figure S4 in File S1), suggesting that Msk does not affect activated Notch in notum myoblasts.

Vg is a known transcriptional target of Wg signaling in the developing wing pouch (Swarup and Verheyen 2012) and may be a direct target of Wg signaling in the AMPs (Sudarsan *et al.* 2001). Few Wg targets have been identified in wing disc notum myoblasts. Thus, we wondered if additional Wg-regulated genes in other cell types also require Msk

function in other populations of adult myoblasts. Maqbool *et al.* (2006) identified Lbe as a target of extrinsic Wg signaling in the developing leg disc (Maqbool *et al.* 2006). Lbe(+) myoblasts were observed in both dorsal and central regions of the leg disc and partially colocalized with the myoblast marker Ebd (Figure 5, G–I; yellow *). Msk reduction by RNAi abolished leg disc myoblast expression of Lbe (Figure 5, J–L). These results suggest a broader role for Msk in general myoblast proliferation and maintenance of the Wg-responsive proteins Vg and Lbe in different tissues.

We next sought to place Msk within the Wg signaling pathway. Ebd is a DNA binding protein that physically interacts with the transcriptional coactivators Arm and TCF in context-specific, Wg-dependent processes, including IFM formation (Benchabane *et al.* 2011; Xin *et al.* 2011). Ebd is present in L3 wing disc-associated AMPs (Figure 6A). While there is a sharp decrease in the number of Ebd(+) myoblasts upon loss of Msk (Figure 6C), the expression of Ebd was not altered (Figure 6B). Next we examined the relationship between Msk and the Wg coactivator TCF. Overexpression of Msk (Msk FL) did not affect the myoblast pool (compare Figure 6E to 1151-Gal4 controls in Figure 1, E, H, and K). Consistent with published results, expression of dominant-negative TCF (DN-TCF) in the AMPs using the 1151-Gal4 driver reduced the overall myoblast number (Figure 6, E and G) (Sudarsan *et al.* 2001). However, the introduction of excess Msk in a DN-TCF background did not alter the size

Figure 3 Blocking Msk function in founder cells reduces both the myoblast pool size and fiber number. (A–D) Maximum projection confocal pictures of L3 wing disc-associated myoblasts. (A) *rp298* expression is present in a subset of myoblasts as visualized by GFP expression. (B–D) The numbers of larval AMPs labeled with anti-Twi is similar in (B) *rp298-Gal4* or (C) *rp298 > GFP RNAi* controls, but reduced in (D) *rp298 > msk RNAi*-expressing myoblasts. Dotted lines denote larval myoblast pool. (E) A bar graph showing a significant reduction in the density of myoblasts (per regions 1600 μm^2) present in the notum of *rp298 > msk RNAi* wing discs compared to controls. (F–O) The consequences of *msk RNAi* knockdown in DLM fibers at (F–J) 20 hr APF or (K–O) 24 hr APF. Fibers are marked by 22C10 (green; *) and fused myonuclei are labeled with Ewg (red). Six fibers are present in (G, H, L, and M) controls, whereas (I and N) *rp298 > msk RNAi* individuals have less than six fibers. (J and O) Bar graphs showing significantly fewer fibers per hemi-segment at (J) 20 hr APF or (O) 24 hr APF. Mean $\pm$ SEM. n.s., not significant. **** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. Bar, 50 μm .

of the AMP pool (Figure 6, F and G), indicating that Msk does not act downstream of TCF.

To test the hypothesis that Msk functions upstream of Wg-responsive transcription, we next examined whether an activated version of Armadillo (*armS10*) could rescue the myoblast deficit resulting from *msk RNAi* knockdown. Expression of *armS10* alone in myoblasts had no effect on the overall myoblast pool (Figure 6H). However, the introduction of activated Arm in a *msk RNAi* background (Figure 6K) partially rescued the total number of myoblasts compared to *msk RNAi* alone (Figure 6I). In contrast, expression of UAS-driven GFP did not alter the number of myoblasts compared to *msk RNAi* alone (Figure 6J), indicating that an additional UAS line did not dilute out the effectiveness of the Gal4 protein.

Quantitation using two different parameters confirmed rescue. Counting both myoblast density (Figure 6L) and the total number of myoblasts in a single plane (Figure 6M) revealed an increase when *armS10* was expressed in a *msk RNAi* background over *msk RNAi* alone or *msk RNAi*, UAS-GFP. Here we conclude that Msk lies upstream of the Arm/TCF transcriptional complex, although these experiments cannot rule out the role of Msk in a parallel pathway.

Proper Msk function is essential for Arm in the myoblast pool

The subcellular localization of Arm plays a pivotal role in transducing Wg signaling. The kinase Sgg/GSK-3 β phosphorylates cytoplasmic Arm and targets the protein for

Figure 4 Knockdown of *msk RNAi* during pupal morphogenesis does not alter the number of myonuclei, but causes a minor delay in fiber formation. (A) Schematic showing the temperature-shift paradigm for *msk RNAi* induction during pupal development. (B–D and F–H) Maximum projection confocal micrographs of DLM fiber formation at 24 hr APF. Fibers are marked by 22C10 (red; *) and fused myonuclei are immunostained with Ewg (green). (B–D) Controls have the normal complement of six fibers. (F–H) A mild decrease in fiber number is observed upon *msk RNAi* knockdown. Note that complete fiber splitting is delayed (G, white ←). (E) Bar graph quantitates the small decrease in fiber number upon a reduction in Msk. (I) Quantitation reveals no difference in the number of myonuclei between (D) control and (H) experimental samples. Mean $\pm$ SEM. n.s., not significant. *** $P < 0.005$. Bar, 50 μ m.

destruction by the proteasome in the absence of Wg ligand (Bejsovec 2006; Swarup and Verheyen 2012). However, in cells that receive Wg signal, Sgg and other members that compose this so-called destruction complex are inactivated; resulting in cytoplasmic Arm accumulation and translocation to the nucleus to activate Wg-responsive genes. If Msk acts upstream of Arm, we may expect to see altered subcellular localization of the Arm protein.

A percentage of myoblasts expressing Arm was observed as a readout of Wg signaling in controls (Figure 7, A–D, arrowheads). The location of these myoblasts appeared stochastic as individual wing discs showed different populations of Arm(+) cells within the proliferating myoblast population. However, abrogation of Msk function nearly eliminated Arm expression in the remaining myoblasts (Figure 7, E–G). Quantitation showed a reduction in Arm(+)/Twi(+) cells from ~20% in control myoblasts to <2% upon decreased Msk function (Figure 7H).

To further examine the relationship between Wg and Msk, we analyzed the distribution of Twi-expressing myoblasts

relative to the source of Wg ligand in the notum epithelial cells. Consistent with previous results (Sudarsan *et al.* 2001), Wg protein was detected in a stripe of cells underlying the myoblast pool (Figure 7, I–K). This is further demonstrated by XZ scans showing myoblasts in a plane above the Wg-expressing epithelial cells (Figure 7L). Analysis of *msk RNAi* wing discs revealed a number of insights. First, Wg protein distribution was not altered upon *msk RNAi* knockdown (Figure 7, M and N). This important observation suggests that Msk does not act in a cell-nonautonomous manner to influence Wg production and/or secretion. Second, the remaining myoblasts in XY-plane views were still present at the most dorsal and ventral regions of the wing disc (Figure 7, O and P; white ←), suggesting that Msk responds to Wg signaling at distant locations from ligand production. Finally, *msk*-depleted myoblasts were also observed in a layer most distal from the site of Wg production (Figure 7P, yellow ←). We conclude from these experiments that there is no correlation between the source of Wg and ability of Msk to respond to Wg signaling to promote myoblast proliferation.

Figure 5 Msk regulates the expression of Wg-responsive genes in the wing imaginal disc and leg disc. (A–F) Myoblasts in L3 wing discs immunolabeled with Cut (red) and Vg (green) in (A–C) controls, compared to those with (D–F) disrupted Msk function (*1151 > msk RNAi*). While both Vg and Cut exhibit broad myoblast expression, (A and B; *) Vg accumulates at higher levels in the dorsal myoblasts while (A and C; ←) Cut protein is seen at increased levels in ventral myoblasts. (D and F) Cut staining is still present, while (D and E) Vg expression is absent upon induction of *msk RNAi*. (G–L) Effect of blocking Msk function on Lbe expression in leg disc-associated myoblasts. (G–I) In control animals, myoblasts are double labeled with Ebd (green) and Lbe (red, *). (J–L) Disruption of Msk function results in a significantly fewer Ebd(+) myoblasts accompanied by loss of Lbe expression. All images are Z-stack projections. Bar, 50 μ m.

The cytoplasmic-to-nuclear translocation of Arm is usually a readout of Wg signaling. Surprisingly, Arm protein was undetectable in *msk RNAi* wing disc-associated myoblasts. To test the hypothesis that Msk may be required for Arm protein stability, we chose to block Wg signaling in the L3 AMPs by overexpressing either WT [UAS-*sgg* (WT)] or an activated version of Sgg [UAS-*sgg* (Y214F)] (Bourouis 2002). Excess *sgg* (WT) significantly decreased the overall myoblast density (Figure 8, B and D) compared to *1151* controls (Figure 8, A and D). Note that this block in Wg signaling was comparable to the overexpression of *DN-TCF* in AMPs (Figure 6, E and G). Overexpression of the activated *sgg*

(Y241F) line, which is thought to retain low levels of kinase activity, also showed a decrease in myoblast density (Figure 8, F and H). This disruption in Wg signaling was less severe compared to the expression of *sgg* (WT), but significant compared to *1151* controls (Figure 8, E and H). Next we tested if overexpression of Msk altered the Sgg-dependent reduced myoblast number. Indeed, driving Msk FL in larval AMPs either partially or fully restored the myoblast pool in a *Sgg* (WT) (Figure 8, C and D) or *Sgg* (Y214F) (Figure 8, G and H) background. Note that expression of Msk alone did not alter myoblast number (Figure 6, D and G and Figure 8, D and H). These data, taken together, suggest Msk may affect the Sgg destruction complex upstream of the Arm/TCF coactivator proteins responsible for Wg transcriptional responses.

Discussion

Drosophila makes two sets of muscles during its life cycle: embryonic body wall muscles required for larval movement and adult muscles necessary for flight, climbing, and mating. An important difference between these two muscle sets is their size. Embryonic muscles are smaller and are comprised of ~2–35 myonuclei (Bate 1990). This final muscle size is largely dependent on the total number of fusion events that occur in myogenesis. In contrast, the muscles that compose one subset of adult muscles, the IFMs, are large and each muscle fiber is made up of ~3000 myonuclei. The small 8–12 cell AMP precursor pool that generates these muscles is set aside in the embryo, remain undifferentiated, and become associated with wing imaginal discs (Bate *et al.* 1991). Rapid proliferation of these AMPs in the later larval and early pupal stages is critical for attaining final muscle cell size. Taken together, these results suggest that IFM myogenesis provides us a unique opportunity to understand factors that regulate AMP pool size and, in turn, muscle formation.

In this study, we demonstrate a new role for Msk in the regulation of AMP number and DLM formation. First, reduced Msk function results in minimal fiber formation, likely a consequence of decreased AMP numbers. In theory, this smaller pool size could be due to reduced proliferation, increased cell death, or premature muscle differentiation. Two possibilities have been ruled out: increased cell death was not observed in the myoblast pool upon Msk reduction, and no indication of premature muscle differentiation was present in *msk RNAi* animals. However, blocking Msk function resulted in a severe depression in myoblast proliferation as assayed by PH3 staining. Thus, our data show that the reduction in myoblasts is largely due to reduced AMP amplification during the normally proliferative larval stages.

Our laboratory and others have obtained evidence that IFM formation is dictated by the size of the AMP pool. Disruption of TCF or overexpression of the Vg-repressor Cut results in a severe depletion in AMP number and minimal DLM fiber development (Sudarsan *et al.* 2001). Our data also show that blocking Msk function in all myoblasts causes a strong reduction in the overall size of the myoblast pool and a lack of fiber

Figure 6 Moleskin acts upstream of Wg transcriptional complexes. (A and B) Maximum projection confocal images of wing disc-associated myoblasts marked by Ebd antibody in control and *msk RNAi* samples. (C) The myoblast density (per regions 1600 μm^2) is significantly less in (B) *msk RNAi* samples compared to (A) controls. (D–G) Effect of overexpressing Msk in a *DN-TCF* mutant background. Myoblasts are marked by Twi in the notum region of L3 wing discs in Z-stack projections. (D) Overexpression of Msk alone does not alter the myoblast pool number. (E) Expression of *DN-TCF* results in reduced density of the myoblast pool. (F) Overexpression of Msk in a *DN-TCF* background does not rescue the reduction in myoblast number. (G) Quantification of the myoblast pool density in the indicated genotypes. (H–K) Effect of overexpressing *armS10* in an *msk RNAi* mutant background in maximum intensity projections. (H) The Twi-labeled myoblast pool in *armS10* wing discs is similar to controls. (I and J) A diminished myoblast pool is present in both (I) *1151 > msk RNAi* and (J) *1151 > GFP; msk RNAi* wing discs. (K) Overexpressing *armS10* partially rescues the myoblast pool size. (L and M) Quantitation comparing the (L) myoblast density (per regions 1600 μm^2) or (M) myoblast pool size per single confocal plane in the indicated genotypes. Mean $\pm$ SEM. n.s., not significant. **** $P < 0.001$, *** $P < 0.005$, ** $P < 0.01$. Bar, 50 μm .

Figure 7 Disrupting Msk function results in loss of Arm protein. (A–G) Immunofluorescent double-labeling of Arm (green) and myoblasts (red) marked by Twi antibody in L3 wing discs. (A–C) Controls show an accumulation of Arm in a fraction of myoblasts depicted as maximum intensity projections. (D) Single plane orthogonal views through the notum wing disc showing colocalization of Twi(+) and Arm(+) myoblasts. (D') Increased magnification of the XZ scan in D. The yellow ← points from the epithelium toward the myoblast layers in the notum. (E–G) There is no Arm accumulation in the remaining myoblasts in *1151 > msk RNAi* wing discs shown as Z-stack projections. (H) Bar graph shows a significant reduction in the fraction of Arm(+) myoblasts upon a reduction of Msk. (I–K) Maximum projection confocal images of L3 wing discs double labeled with Wg (green) and Twi (red). (L) A single plane orthogonal section of the wing disc showing Wg-expressing epidermal cells and the overlying myoblasts. (L') Increased magnification of the XZ scan in L. The yellow ← points away from the source of Wg toward the myoblasts. (M–O) Similar to controls, the myoblast pool (red) in *msk RNAi* animals is

Figure 8 Msk acts through Sgg to regulate the wing disc myoblast pool size. (A–C and E–G) Maximum confocal projections of L3 notum myoblasts immunostained with Twi. (A and B) Qualitatively, less myoblasts are present upon overexpression of (B) *sgg* (WT) compared to (A) *1151* controls. (C) Overexpressing Msk in a *sgg* (WT) background partially rescues the myoblast number. (D) Quantification of myoblast density (per regions 1600 μm²) in (A–C). (E and F) Targeting a weak version of (F) activated *sgg* (Y214F) causes a reduction in the myoblast pool compared to (E) *1151* controls. (G) A significant increase in the myoblast pool size is seen in *1151 > msk FL; sgg* (Y214F) samples. (H) A bar graph showing partial restoration of the myoblast pool (per regions 1600 μm²) upon overexpression of *msk FL* in a *sgg* (Y214F) background. Mean ± SEM. n.s., not significant. **** $P < 0.001$, *** $P < 0.005$. Bar, 50 μm.

formation. However, the effect on the AMP population and DLM number is less severe when Msk function is reduced only in founder myoblasts. Similarly, blocking Rac1 GTPase activity in notum myoblasts causes only a minor reduction in AMP number and a slight reduction in fiber number (Fernandes *et al.* 2005). This strong correlation between cell proliferation and organ size has been an emerging theme in multiple development systems (Breuninger and Lenhard 2010; Tumaneng *et al.* 2012).

Msk is a member of the β-like importin family of proteins and is most similar to mammalian Importin-7 (53% identity and 71% similarity to mouse Imp-7). The canonical role of the β-like importin family is in the nuclear import of proteins in response to extracellular stimuli (Flores and Seger 2013). This import can be dependent or independent of cargo containing a classical NLS and sometimes requires a physical interaction with importin-β proteins. A number of cargoes have been identified that require Imp-7 for nuclear translocation. Some are general cellular proteins, such as ribosomal

proteins (Jäkel *et al.* 1999; Fassati *et al.* 2003; Freedman and Yamamoto 2004), while others are transcription factors that are imported in response to stimulation, including both vertebrate and *Drosophila* ERK (Lorenzen *et al.* 2001; Michailovici *et al.* 2014). However, we did not observe an effect of Msk reduction on diphosphorylated ERK (dpERK) subcellular localization or protein levels in the myoblasts (Figure S4 in File S1). Moreover, RNAi knockdown of the *Drosophila* Importin-β homolog Ketel, which is required for the nuclear import of dpERK in embryos (Lorenzen *et al.* 2001), did not reduce the AMP pool in notum myoblasts (Figure S5 in File S1). Together, our results suggest that Msk and Ketel do not function together in AMP proliferation, but highlight the importance of a novel role for Msk in response to external Wg as a signaling stimulus.

Where does Msk fit within the known paradigm of *Drosophila* AMP proliferation and IFM formation? A study by Gunage *et al.* (2014) shows that the initial postembryonic amplification of AMPs in L2 larvae is regulated by the Notch

evenly distributed relative to the Wg(+) (green) cells. Also, there is no difference in Wg staining between the control and the experimental samples. (P) Orthogonal section of notum wing discs show that the myoblasts are juxtaposed next to a source of Wg, but maintain their location at the distal edge of the myoblast layer. (P') Increased magnification of the XZ scan in P. The yellow ← points away from the source of Wg toward the myoblasts. Note that two different samples are shown in (M) and (P). In all orthogonal views, the upper panel corresponds to an XZ view of the red line and the green line is the location of the XZ view of the green line. Mean ± SEM. **** $P < 0.001$. Bar, 50 μm.

pathway, and further AMP proliferation is under control of Wg signaling in L3 larvae (Gunage *et al.* 2014). Furthermore, secreted Wg acts through TCF in the AMPs for the maintenance of Vg expression in myoblast proliferation and subsequent IFM muscle formation (Sudarsan *et al.* 2001). Since our data show that Vg protein is absent upon a reduction in Msk function, we reasoned that Msk may be responsive to Wg signaling. The introduction of excess Msk does not rescue decreased myoblast proliferation due to a block in Wg signaling by DN-TCF. However, activated Arm partially rescues the myoblast deficit resulting from *msk RNAi*. Collectively, these results place Msk upstream or parallel to TCF and Arm to regulate myoblast pool size through Vg.

Our findings suggest that Msk may regulate the myoblast pool size by two possible mechanisms, which are not mutually exclusive. First, Msk may directly regulate the nuclear import of Arm and/or TCF in response to Wg stimulation. A second possibility is that Msk may control the stability of Arm in the cytoplasm. Excess levels or activation of Sgg, one of the components of the Arm destruction complex (Apc/Axin/Sgg), reduces AMP pool size. This result suggests that Arm stability is critical for myoblast amplification. Interestingly, overexpressing Msk in combination with excess or activated Sgg partially rescues the myoblast pool. Taken together, our results support the idea that Msk might regulate myoblast pool size by controlling Arm stability through Sgg. Our future experiments will be aimed at examining if Msk biochemically interacts with destruction-complex proteins.

Does Msk regulate Wg signaling which controls myoblast pool size in other muscle groups? Leg muscles are derived from a population of myoblasts associated with imaginal leg discs. One of the known Wg targets, *Lbe*, is expressed widely in leg disc myoblasts and is known to regulate muscle growth and performance (Maqbool *et al.* 2006). Loss of Wg signaling results in the loss of *Lbe* expression in the leg disc myoblasts, which in turn leads to impaired muscle patterning. Our data show that Msk dictates myoblast pool size in the leg disc. Similar to Wingless loss of function, blocking Msk results in the absence of Wg target *Lbe* in the leg disc myoblasts. Collectively, these results suggest that Msk might function as a general regulator of Wg signaling during muscle formation.

The data here increase our general understanding of stem cell regulation, subsequent organ formation, and patterning during development. Furthermore, since AMPs phenocopy some features of vertebrate satellite cells, our findings may provide insight into mechanisms regulating satellite cell proliferation following muscle injury in vertebrates. Our next step is to examine whether Msk regulates the amplification of myoblast pool size during muscle injury or aging.

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