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Downstream of identity genes: Muscle-type specific regulation of the fusion process

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Abstract

In all metazoan organisms, the diversification of cell types involves determination of cell fates and subsequent execution of specific differentiation programmes. During *Drosophila* myogenesis, identity genes specify the fates of founder myoblasts, from which derive all individual larval muscles. Here, to understand how cell fate information residing within founders is translated during differentiation, we focus on three identity genes, *eve*, *lb* and *slou* and how they control the size of individual muscles by regulating the number of fusion events. They achieve this by setting expression levels of *Mp20*, *Pax* and *mspo*, three genes that regulate actin dynamics and cell adhesion and, as we show here, modulate the fusion process in a muscle-specific manner. Thus, these data provide the first example of how the identity information implemented by transcription factors is translated *via* target genes into cell-type specific programmes of differentiation.

Keywords

Identity genes; fusion; muscle; Drosophila; Mp20; Paxillin; M-spondin

INTRODUCTION

Correct diversification of cell types and determination of unique properties of cells in a tissue is crucial for the harmonious progression through development and formation of functional organs. In a broad range of developing tissues and metazoan organisms, diversification of cell fates is controlled by a set of transcription factors encoded by cell identity genes (e.g. Carmena et al., 1998; Halfon et al., 2000; Dalla Torre di Sanguinetto et al., 2008). A large number of key transcriptional regulators promoting diversification of cell types have been identified in *Drosophila* (Skeath, 1999; Baylies and Michelson, 2001; Olson, 2006), however our understanding of the gene expression programme that operates downstream of identity genes and leads to the acquisition of specific cell properties remains very limited.

Particularly well suited for studying diversification of cell types is the *Drosophila* embryonic musculature, composed of a set of morphologically distinct muscles, each of which displays specific properties, such as shape, size, position, innervation and attachment points (Bate, 1990). Each muscle, constituted of one fibre, arises from a specialized myoblast called muscle founder cell (FC). Specification of individual FCs is determined by a combinatorial code of muscle identity genes (Frasch, 1999) including those that are the

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focus of this work: *ladybird* (*lb*) (Jagla et al., 1998), *S59/slouch (slou)* (Knirr et al., 1999) and *even-skipped* (*eve*) (Su et al., 1999). Loss of function or ectopic expression of identity genes transforms the fate of FCs, causing an aberrant muscle pattern (e.g. Ruiz-Gomez et al., 1997; Knirr et al., 1999). However, despite significant progress in understanding FCs specification, the molecular mechanisms underlying the progression from FC to a mature muscle having unique properties remains poorly understood.

To date only a few large-scale approaches have been used to identify genes expressed in FCs and differentiating muscle (Artero et al., 2003; Estrada et al., 2006; Sandmann et al., 2006; Junion et al., 2007). Among them is our previous work identifying downstream targets of Lb (Junion et al., 2007). This revealed that during muscle development *lb* acts at multiple levels as it not only contributes to the combinatorial code of transcription factors specifying the FCs, but also regulates a large number of genes involved in setting cell shape, adhesion, and motility. These data suggest that by regulating a set of targets, the identity genes could control muscle-type specific programmes of differentiation.

To test this possibility we decided to focus on one of the earliest events of muscle differentiation, the regulation of myoblast fusion which leads to the formation of syncytial myotubes with an appropriate size. Fusion is a reiterative process that occurs during embryonic stages 12-15 (Beckett and Baylies, 2007) between FCs and a pool of Fusion Competent Myoblasts (FCMs). The number of fusion events differs from muscle to muscle, so that at the end of myogenesis, some muscles contain only 4 nuclei whereas others can contain up to 25 nuclei (Bate, 1990). A large number of genes required for fusion (Abmayr et al., 2008) have been identified, however, all of them act in all muscles and the mechanisms regulating number of fusion events in muscle-type specific manner remain unknown.

Given that the individual FCs are specified to generate a muscle of a particular size, one possibility is that the identity genes control the fusion counting. Here, to test this hypothesis we focus on the fusion process in 5 muscles that require the identity genes *eve*, *lb* and *slou* (Jagla et al., 1998; Knirr et al., 1999; Su et al., 1999). We show that these identity genes are indeed able to control the number of fusion events. They do this by regulating the expression of regulators of actin cytoskeleton and cell adhesion, *Muscle protein 20 (Mp20), Paxillin (Pax)* and *m-spondin (mspo)*, previously identified as Lb targets (Junion et al., 2007). Eve, Lb and Slou induce specific combinations of *Mp20*, *Pax* and *mspo* levels, which in turn determine specific programmes of fusion. Thus, our data provide direct evidence for the role of muscle identity genes in fusion counting and, more generally, illustrate how a combinatorial identity code is translated into a combinatorial realisator code of identity gene targets that execute cell type diversification.

RESULTS

Availability of FCMs has no impact on muscle-specific fusion counting

Myoblast fusion takes place between two types of cells: the FCs and the FCMs. Although it was shown that each cell type plays a crucial role in the fusion process (Ruiz-Gomez et al., 2000; Beckett and Baylies, 2007; Kocherlakota et al., 2008), the role of each cell type in the control of the number of fusion events in a muscle-type specific manner is unknown. Two distinct models can be proposed to explain how muscle-specific fusion counting is regulated: i) FCs and a pre-defined number of FCMs are specified locally to determine the size of resulting muscles; or, ii) only FCs control the number of fusion events whereas FCMs are not limiting. To analyse the muscle-type specific regulation of the fusion, we focused our analyses on five muscles: DA1, SBM and DT1, VA2, VT1. Following fusion, the new nuclei provided by the FCMs turn on the identity genes expressed in the original

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FC, and therefore we can use antibodies detecting Eve, Lb and Slou to count nuclei in DA1, SBM and DT1, VA2, VT1 muscles, respectively.

First, to test if FCM number is limiting during fusion process we overexpressed the attractant protein Dumbfounded (Duf) (Ruiz-Gomez et al., 2000) in all FCs using *duf-Gal4*. This led to an increased number of nuclei in all muscles analyzed (Figures 1A, Figure S1 and Table S1, Table S2) suggesting that FCMs number is a non-limiting factor during myoblast fusion. Second, to further investigate whether FCMs could influence number of fusion events, we manipulated two adjacent muscles, SBM and VA2 (Figure 1A, 1B). We first sought to reduce the number of free FCMs around SBM by increasing the number of VA2 fusion events. This was achieved by overexpressing Duf in the VA2 FC (*slou-Gal4; UAS-duf*) (Figure S1, Table S2). Even though this should reduce the number of nearby FCMs, there was no change in SBM nuclei number (Figure 1A). Then we performed the reciprocal experiment, increasing FCMs around SBM, by inducing apoptosis of the VA2 FC (*slou-Gal4; UAS-rpr*). This also did not change the nuclei number in SBM. Similarly, VT1, VA2 and SBM were not affected when we destroyed or increased the size of VL1 (Figure S1, Table S2). Thus, even if we cannot exclude some compensatory proliferation of FCMs (Beckett and Baylies, 2007), the induced changes of FCM number did not influence fusion counting by adjacent muscles, favouring the second model where the identity information carried within FCs specifies how many FCMs fuse with each FC.

Fusion counting is regulated by the identity genes in a muscle-specific manner

Identity genes have been shown to be required for FC specification, however, their capacity to regulate the number of fusion events has never been analyzed. Each muscle whose formation depends on specific identity gene contained a consistent number of nuclei: *eve*/DA1 has 11 nuclei; *lb*/SBM 7; and the *slou* dependent DT1, VA2 and VT1 muscles contain respectively 8, 9 and 4 nuclei (Figure 1M and Table S1). To investigate whether Eve, Lb and Slou activity specifies the number of fusion events, we have modified their expression using a gain-of-function approach with *duf-Gal4*, expressed in all FCs after their specification, and two FC-specific drivers, *slou-Gal4* and *eme-Gal4* (Figure 1D-1M, Figure S1 and Table S1, Table S3). The overexpression of identity genes in the FCs that normally express these genes (*eve*/DA1, *lb*/SBM and *slou*/VT1) did not alter the nuclei number in the resultant muscles (Figures 1M). This demonstrates that the level of identity gene expression in a muscle in which it is normally acting does not influence fusion counting. However, ectopic expression of *eve* modified counting in DT1 and VA2, driving them toward the DA1 number (Figures 1M). In this case, the ectopically expressed *eve* represses endogenous *slou* expression (Figure 1F). In a similar manner, misexpression of *lb* leads to repression of *eve* and *slou* and the execution of an SBM-like *lb*-programme of fusion, resulting in formation of DA1, DT1 and VA2 having 7 nuclei (Figures 1H, 1I, 1M). Finally, misexpression of *slou* in the SBM represses endogenous *lb* expression and reduces nuclei number to 4, similar to VT1 (Figure 1L, 1M). Since different fusion programmes are induced, by ectopic expression of *lb versus slou* in DA1, even though both repress *eve*, our data rules out an indirect effect of repression.

Altogether, these data demonstrate that identity genes control muscle size by specifying the number of fusion events: Eve specifies recruitment of about 10 nuclei, Lb 6 nuclei, and Slou appears as a key determinant of fusion programme involving 3 fusion events (VT1 muscle). However, other muscles expressing *slou* have a different nuclei number (VA2 and DT1) (Figures 1M), leading us to hypothesize that other identity genes co-expressed with *slou* contribute to fusion counting in these muscles. Thus, these data demonstrate that identity genes are sufficient to specify muscle size by regulating fusion counting.

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Identification of identity genes targets potentially involved in fusion counting

To understand how identity genes exert their function to control the number of fusion events, we sought to identify target genes that may have a role in fusion counting (Figure 2). First, by screening the BDGP *in situ* database (www.fruitfly.org/cgi-bin/ex/insitu.pl) we identified 31 candidates with an expression pattern restricted to muscle subset consistent with a potential regulation by identity genes. Among them, we selected candidates with GO annotations suggesting an involvement in cell adhesion or cytoskeletal regulation, which could have a role in myoblast fusion, resulting in 9 genes (Table S4). Finally, 3 of these genes are also Lb targets (Junion et al., 2007): *Muscle Protein 20* (*Mp20*), *Paxillin* (*Pax*) and *m-spondin* (*mspo*).

Mp20, *Pax* and *mspo* display muscle-type specific expression levels

To characterise in detail *Mp20*, *Pax* and *mspo* expression patterns during myogenesis we used fluorescent *in situ* hybridizations combined with antibody staining for general and muscle-specific markers. Each of candidate genes is indeed expressed in a muscle-type specific manner but also in other tissues (Figure 3 and Figure S2). Their expression starts at stage 13 and transcripts can be detected in muscles throughout the muscle fusion period, until stage 15 (Figures 3 and Figure S2). Co-labelling with *duf-LacZ* showed that *Mp20*, *Pax* and *mspo* are detectable in muscles growing by fusion, but not in unfused FCMs (Figure 3D, 3I, 3N). These 3 candidates showed different levels of expression in our muscles of interest (DA1, DT1, SBM, VA2 and VT1). *Mp20* is expressed at a high level in VA2 and DT1, at a median level in SBM, at low levels in DA1 and is not detectable in VT1 (Figure 3A-3C'). *Pax* is expressed at a high level in VA2, at a median level in DT1, and at low levels in SBM, DA1 and VT1 (Figure 3F-3H'). Finally, *mspo* transcripts accumulate at a high level in the DT1, at a median level in VA2, a low level in DA1 and VT1 and are not detectable in SBM (Figure 3K-3M').

To summarize this data we have employed a colour intensity code representing the expression levels (high, median and low) for each candidate gene (Figure 3E, 3J, 3O). This analysis shows that each of the muscles we are focusing on has a unique signature provided by differing expression levels of the three candidates.

Expression level of *Mp20*, *Pax* and *mspo* is regulated by identity genes

Differential muscle-specific expression of *Mp20, Pax* and *mspo* and the fact they were identified as Lb targets (Junion et al., 2007) strongly suggest that identity genes regulate their transcription. To confirm this, we induced ectopic expression of *eve*, *lb* and *slou* with the panFC *duf-Gal4* driver and checked whether they alter expression patterns of *Mp20*, *Pax* and *mspo*. PanFC *eve* induced a low expression level of the three candidates in all muscles (Figure 4B, 4F, 4J and Figure S3) similar to that normally found in the *eve*+ muscle DA1. Similarly, panFC *lb* and *slou* induce at ectopic positions the combination of expression levels of *Mp20*, *Pax* and *mspo* observed respectively in *lb*+ SBM (Figure 4C, 4G, 4K and Figure S3) and in the *slou*+ VT1 muscle (Figure 4D, 4H, 4L and Figure S3). The ectopic *slou* can also alter *Mp20*, *Pax* and *mspo* expression levels in a different manner, that resembles to the combinations found in other *slou*+ muscles, VA2 and DT1. Taken together, our data show that not only *lb*, but also *eve* and *slou*, control the muscle-specific expression levels of *Mp20*, *Pax* and *mspo* and are required for setting the signature patterns of their expression in different muscles (Figure 4M).

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Differential expression levels of *Mp20*, *Pax* and *mspo* regulate fusion counting in a muscle specific manner

To test the function of *Mp20*, *Pax* and *mspo* we used muscle specific knockdown *via* RNAi (*Mp20, Pax*) or if available (*mspo*), specific null mutation. Furthermore, we generated a molecular null allele, *Pax*$^{\Delta 1}$ that deletes almost all of the *Pax* coding region, the downstream gene *CG31798*, and one of two *CG17544* transcripts (Figure 5A). Animals homozygous for these mutations die as pupae, but survived when rescued by a transgene construct encoding a GFP-tagged version of the *Pax* gene, showing that the lethality is caused by the absence of *Pax* (Figure S4).

As fusion defects are manifested by the presence of unfused myoblasts, we first analysed late stage embryos lacking *Mp20*, *Pax* and *mspo* function. An increased number of unfused cells was detected with anti-ß3-Tub and with anti-MHC stainings in *Mp20* RNAi, *Mp20* Df(2R)Exel7124 deficiency, *Pax* RNAi and *Pax* null mutant embryos (Figure 5 and Figure S4) but not in homozygous *mspo* mutants (Figure 5) suggesting that at least *Mp20* and *Pax* are involved in fusion.

To check whether there is a link between differential expression of *Mp20*, *Pax* and *mspo* in muscles and their function in myoblast fusion we analyzed nuclei number in our muscles of interest (Figure 6A and Table S5). RNAi knockdown of *Mp20* decreased nuclei number by about 2 in muscles DT1 and VA2 that express *Mp20* at high levels, while a lower decrease is found in SBM, which has median *Mp20* levels, and there was no change in muscles with low or absent *Mp20*, DA1 and VT1. In a similar manner, *Pax* RNAi attenuation or the *Pax* mutation caused loss of nuclei from muscles expressing a high or median *Pax* levels, VA2 and DT1, and no change in DA1, SBM and VT1 muscles with low *Pax*. Importantly, rescue experiments of *Pax*$^{\Delta 1}$ deficiency showed that *Pax-GFP* restored the normal fusion programme in affected muscles, whereas the rescue with a genomic fragment encompassing the PDLP isoform, a truncated form of Pax encoded by the same genomic locus (Yagi et al., 2001), did not (Figure S5 and Table S5). In contrast to *Mp20* and *Pax*, *mspo* loss of function increased nuclei numbers. This was observed in only one muscle (DA1), with relatively low *mspo* expression. The finding that *mspo* acts as a negative regulator of myoblast fusion is consistent with the lack of unfused cells in *mspo* mutants (Figure 5D). Altogether, the loss of function analyses show that the muscle-specific levels of *Mp20*, *Pax* and *mspo* plays instructive roles in setting the number of fusion events (Figure 6B).

To further investigate the role of differential expression of *Mp20*, *Pax* and *mspo* during myoblast fusion we tested the effect of their overexpression. We used *eme-Gal4* to overexpress *Mp20*, *Pax* and *mspo* in DA1 and *slou-Gal4* for overexpression in DT1, VA2 and VT1 (Figure 6C-6I, Figure S5 and Table S6). *Mp20* overexpression increased nuclei number in DA1 but not others (Figure 6C, 6E and Figure S5). The inability of *Mp20* overexpression to increase nuclei numbers in DT1 and VA2 could be due to a saturation effect due to their high endogenous *Mp20* levels. Similarly, *Pax* overexpression increased nuclei number in DA1, DT1 and VT1, whereas VA2, with a high endogenous *Pax* level, was not affected (Figure 6C, 6F and Figure S5). In contrast, *mspo* overexpression decreased nuclei number, notably in DA1 (Figure 6C, 6G and Figure S5). Altogether these data show that altering the expression level of *Mp20*, *Pax* and *mspo* results in a proportional modulation of the number of fusion events.

Finally, we asked how *Mp20*, *Pax* and *mspo* act together during modulation of myoblast fusion. First, we ruled out the possibility that *Mp20*, *Pax* and *mspo* are interdependent by analyzing their expression in null mutants for each of them (Figure S6). Then, we performed double loss and gain of function experiments. The double RNAi against *Mp20* and *Pax* induced a strong decrease in nuclei number in DT1 and VA2, two muscles affected by single

RNAi knockdowns (Figure 6A). In contrast, SBM, in which *Pax* seems not to be functional, is affected at the same level in double *Pax/Mp20* and in single *Mp20* RNAi contexts (Figure 6A). To test effects of combinatorial over-expression, we analyzed DA1, whose fusion programme is affected by overexpression of each candidate. The double *Mp20/Pax* gain of function induces a strong increase in nuclei number, much higher than single gain of function of *Mp20* or *Pax* (Figure 6C, 6H compare to 6E, 6F), whereas overexpression of *Mp20* and *mspo* or *Pax* and *mspo* restored a wild type DA1 nuclei number (Figure 6C and 6I compare to 6D, 6E, 6G). Thus, *Mp20, Pax* and *mspo* have independent and additive functions in modulation of fusion programmes and contribute to the acquisition of specific properties of muscles.

Identity genes and their targets regulate muscle-specific number of fusion events by modulating fusion rate

In order to better understand how the muscle specific fusion counting takes place, we have analysed the kinetics of fusion in DA1, SBM, VA2 and VT1 muscles in wild type context by counting number of nuclei at five time points: at embryonic stage 12, 13, 14, early 15 and late 15. As previously reported by Beckett and Baylies (2007), the majority of fusion events occur in all muscles between stage 13 and 15. Our data (Figure 6 J) show that at stage 13, each of four analysed muscle precursors contain about 2 nuclei and that starting from this point each muscle displays specific rate of fusion, which is linear between stage 13 and 15. The fusion rate is low in VT1 and progressively higher in SBM, VA2 and DA1 suggesting that during the second phase of fusion the rate is proportional to the final size of the muscle. Importantly, an ectopic expression of Lb in the DA1 is sufficient to change the DA1 fusion rate to a SBM-like one showing that the kinetics of fusion is regulated by the identity genes (Figure 6J). To determine whether the identity gene targets also modulate fusion process by accelerating or slowing down its rate we analysed effects of attenuation or overexpression of *Mp20, Pax* and *mspo* on kinetics of fusion. The attenuation of *Mp20* and *Pax* leads to a reduced fusion rate measured in VA2 (Figure 6K) whereas their overexpression results in accelerated fusion in DA1 compared to the wild type (Figure 6L). In contrast, the overexpression of *mspo*, acting as a negative regulator of fusion, slows the fusion rate down (Figure 6L). Altogether the analyses of kinetics of fusion provide first insights into mechanistic understanding of muscle-type specific regulation of fusion process revealing that the identity genes and their targets regulate the number of fusion events by setting fusion rate.

DISCUSSION

Development of metazoan organisms is based on acquisition of distinct cell identities so that diverse cell types are generated. During *Drosophila* myogenesis, muscle cells undergo diversification process to form a set of muscles each of which displays a particular size, shape and orientation. It has been demonstrated that the acquisition of muscle identity is initiated by the identity gene-dependent specification of muscle FCs, however, how this identity information is translated into specific properties of resulting muscles remained unknown. Here, to fill this gap we analyse roles of identity genes in regulation of myoblast fusion and identify their targets whose function allow the execution of muscle-specific programmes of fusion.

FCMs do not carry information about muscle-specific fusion programmes

The myoblast fusion is asymmetric and takes place between FCs and FCMs. Previous reports (Estrada et al., 2006; Beckett and Baylies, 2007; Beckett et al., 2008) were at the origin of a hypothesis that FCMs are not “naïve” myoblasts and contribute to the modulation of fusion process. In contrast, our results support a view that FCs rather than FCMs carry the

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instructive information and allow us to conclude that FCMs do not play an active role in setting the number of fusion events. However, because the spatial distribution of FCMs seems to be non uniform (Beckett and Baylies, 2007) it is conceivable that the local distribution of FCMs was coordinated with the requirements of FCs to facilitate fusion process.

Identity information transmitted by identity genes to FC cells modulates fusion programmes

The identity genes *lb*, *slou* and *eve* are required to specify FCs at the origin of five muscles the DA1, DT1, SBM, VA2 and VT1 (Jagla et al., 1998; Knirr et al., 1999; Su et al., 1999). Here we provide evidence that these identity genes are also required for setting the muscle-specific number of fusions and demonstrate how this identity information is executed (Figure 7). After specification step, FCs fuse, between the embryonic stage 12 and 15, with a determined number of FCMs to generate muscles with a specific number of nuclei. During this time period *eve*, *lb* and *slou* continue to be expressed in subsets of developing muscles and our data show that they are sufficient to establish the muscle-specific fusion programmes in DA1, SBM and VT1 (11, 7 and 4 nuclei respectively). Furthermore, *slou* in combination with other factors (see below) contributes to two other programmes that end up with 7 to 8 fusion events in muscles DT1 and VA2. To regulate number of fusion events *eve*, *lb* and *slou* act by modulating expression of genes involved in dynamics of actin cytoskeleton or cell adhesion. Starting from stage 13, they establish a muscle-specific combinatorial code of expression levels of three targets: *Mp20*, *Pax* and *mspo*. The combination of expression of the targets leads to the muscle-specific control of the number of fusion events. This notion is supported by the fact that each of identity genes is able to impose at ectopic locations the combinatorial realisator code of *Mp20*, *Pax* and *mspo* expression, and thus, ectopically execute its fusion programme (Figure S7). Given that the code of *Mp20*, *Pax* and *mspo* is not sufficient to explain fusion programmes in all muscles, we hypothesize that other identity gene targets exist that modulate fusion counting.

Moreover, our data are in support of the two-step model of myoblast fusion according to which a muscle precursor is formed between stage 12 and 13 by an initial fusion, and then, between stage 13 and 15, fuses with additional myoblasts until the muscle reaches its final size (Schroter et al., 2004). The fact that *Mp20*, *Pax* and *mspo* are expressed from stage 13 suggests that the transition point between the two steps depends not only on the timing of FCM migration (Beckett and Baylies, 2007) but also on the activation of limiting factors such as the identity gene targets which modulate the number of additional fusions. As we did not observe nuclear divisions in FCs nor in growing myotubes in all genetic contexts analysed (data not shown) we are confident that the number of nuclei present in each muscle is determined only by the number of fusion events.

Mode of action of identity genes

Specification of FCs requires combinatorial code of activities of identity genes (Frasch, 1999). Here we show that the same identity genes play instructive roles in subsequent muscle-type specific differentiation process. Importantly, our data enlighten the fact that the identity genes are not equivalent and have distinct, context-dependent mode of action. *eve*, *lb* and *slou* are sufficient to set the fusion programmes in DA1, SBM and VT1 muscles, however in VA2 and DT1 *slou* functions in a different way and seems not to have a decisive role in this process. Because the specification of the VA2 and DT1 FCs also involves functions of *Poxm*, *Kr* and *ap* (Ruiz-Gomez et al., 1997; Duan et al., 2007), we hypothesize that they act together with *slou* in setting fusion programmes of VA2 and DT1. This raises an important question about hierarchy of identity genes during execution of muscle identity

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programmes and their roles in acquisition of specific properties of muscles such as number of nuclei, attachment points and innervation.

Mp20, *Pax* and *mspo* act in execution of muscle-specific fusion programmes

The data presented here demonstrate that the number of fusion events in developing muscles is regulated by a muscle-specific combinatorial realisator code of identity gene targets. In contrast to the previously identified fusion genes (e.g. Ruiz-Gomez et al., 2000; Chen and Olson, 2001; Rau et al., 2001) acting in all muscles, the identified identity gene targets, *Mp20*, *Pax* and *mspo*, display muscle-type specific expression and modulate fusion in a muscle-type specific manner proportionally to the level of their expression. The loss and gain of function of each of them lead to subtle fusion phenotypes indicating that the range of fusion events controlled by these three candidates is limited. Indeed, the loss of function of *Mp20* results in loss of 2 nuclei in a subset of muscles whereas its over-expression induces the recruitment of maximum 2 FCMs. A similar range of defects in number of fusion events is observed in *Pax* and *mspo* mutant embryos indicating that they influence fusion process at the same level.

Mp20 encodes a cytoskeletal protein displaying restricted expression in adult muscles (Ayme-Southgate et al., 1989) and sharing sequence homology with the lineage-restricted mouse proteins SM22alpha, SM22beta and NP25. These proteins contain calponin-like repeats, and, in mammals, interact with F-actin and participate in the organization of the actin cytoskeleton (Takahashi and Nadal-Ginard, 1991; Zhang et al., 2002; Mori et al., 2004; Han et al., 2009). In *Drosophila* S2R cells, the RNAi knockdown of *Mp20* induces a phenotype of round and non-adherent cells (Kiger et al., 2003) supporting its role in regulation of fusion process.

The second candidate, *Pax* (*DPxn37*), is a scaffold protein that recruits structural and signalling molecules to the sites of focal adhesion. Pax has been shown to be involved in the actin cytoskeleton organization, cell adhesion, cell migration and cell survival (Hagel et al., 2002; Deakin and Turner, 2008). In the developing *Drosophila* muscles, Pax protein localizes at muscle-tendon junctions (Yagi et al., 2001; Subramanian et al., 2003) (Figure S4) suggesting that it may play a role in muscle attachment. Our analyses of *Pax* mutant embryos do not reveal muscle-tendon adhesion defects, but show discrete myoblast fusion phenotypes, which correlate with differential muscle-specific expression of *Pax*. The role of Pax in modulating fusion is consistent with previously described implications of Pax interacting proteins, including ARF6 in myoblast fusion in both *Drosophila* and vertebrates, and FAK in vertebrates (Tachibana et al., 1995; Brown et al., 1996; Chen et al., 2003; Randazzo et al., 2007; Pajcini et al., 2008; Quach et al., 2009).

Finally, *mspo* belongs to the F-Spondins, a conserved family of ECM proteins, which maintain cell-matrix adhesion in multiple tissues (Feinstein and Klar, 2004). In vertebrates, F-Spondins have context dependent effects on axon outgrowth and cell migration (Schubert et al., 2006). As Mp20, Pax and Mspo are expressed in FC cells and growing myotubes one possibility is that they modify the spreading and/or motility of FC protrusions required to attract FCMs. Alternatively, by modulating actin cytoskeleton Mp20, Pax and Mspo may also influence the stability of adhesion between the growing muscle and the FCM creating permissive conditions or blocking the progression of fusion process.

Muscle-type specific regulation of fusion process by modulating fusion rate

The muscle-type specific regulation of fusion programmes by the identity genes and their targets raises an intriguing question how this regulation is executed from the mechanistic point of view. Because different levels of expression of *Mp20*, *Pax* and *mspo* correlate with

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different fusion programmes in both wild type and genetically manipulated embryos, we thought that by following kinetics of fusion in small and big muscles we may gain insights into how the fusion programmes are modulated. It turns out that the rate of fusion is proportional to the size of muscle, meaning the number of fusion events, thus revealing that the identity genes acting via their targets set up the frequency of fusion events. Accordingly, loss and gain of function of identity genes and their targets identified here results in modulations of fusion programmes by accelerating or slowing down the fusion rate. This finding provides first insights into mechanistic understanding of muscle-type specific regulation of fusion process and raises an important question about whether this mechanism is broadly conserved.

EXPERIMENTAL PROCEDURES

Fly stocks

All *D. melanogaster* stocks were grown on standard medium at 25 °C. The following strains were used: *duf-LacZ* (*rp298-LacZ*; from A. Nose, Univ. Tokyo, Japan), *duf-Gal4* (gift of K. Vijayraghavan, TIFR, India), *UAS-lbe* (Jagla et al., 1998), *UAS-slou* and *slouch-Gal4* (from M. Frasch, Univ. Enlargen, Germany), *UAS-duf* (Ruiz-Gomez et al., 2000), *eme-Gal4* (Han et al., 2002) and *UAS-eve* (gift of R. Bodmer, Burnham Institute, USA), *mspo*^{c26} and *UAS-mspo* (Umemiya et al., 1997), *UAS-Pax* (from G. C. Chen, Academia Sinica, Taiwan). Df(2R)Exel7124, P{*GawB*}*5053A* and P{*GawB*}*how*^{24B} were obtained from the Bloomington Drosophila Stock Center. The *UAS-Mp20RNAi* Ref. 4696R-4, *UAS-PaxRNAi* Ref. 18061R-2 and *UAS-slouRNAi* Ref. 6534R-1 lines come from the NIG-Fly collection. Double mutants *UAS-Mp20RNAi; UAS-PaxRNAi* and *UAS-Pax; UAS-Mp20* or *UAS-Mp20, UAS-mspo* were generated by standard genetic crosses. All *UAS-RNAi* lines were crossed with the early and strong *24B-Gal4* driver to maximize the attenuation effect. Mutants were balanced using CyO, P{*wg*^{en11} *LacZ*} or TM3, *Ser*, P{*twi-LacZ*} and homozygotes were identified by the absence of LacZ staining. *In situ* hybridization or immunochemistry against the transgenes were used to genotype the embryos in gain of function contexts. Crosses and embryo collections were performed at 25°C.

Generation of *UAS-Mp20*

The *UAS-Mp20* construct was made by subcloning a 555 bp NotI-KpnI fragment containing the entire *Mp20* ORF into the pUAST transformation vector (Brand and Perrimon, 1993). Primers used for PCR cloning: 5′-ATAGCGGCCGCATGTCTCTTGAGCGTGCCG-3′ and 5′-GGTACCATATTACTTGCCGAGCAGGATC-3′.

Generation of *Paxillin* mutants

Deletions between EY00742 (P{EPgy2}Pax^{EY00742} BDGP) and EP12861 (DGRC Kyoto #204330) were induced on a stock containing both P elements in transheterozygote and the transposase *Hop6*. From the progeny 550 *white* males were selected, crossed to *w; Bl/CyO* females and then used for a PCR test for the deletion between the two P elements with the primers AGCGTTTGGCTAAGATCGCAGTCGTTCTAT and GATTTAATGTTTCTACATTTGGGATTTTA. Two deletions were identified and recombined with *FRT40A* for induction of mosaic clones.

Rescue experiments

Genomic fragments for the downstream genes (BAC2), *PDLP* and *Pax* rescue constructs were subcloned from BACR28G24 (BDGP, ordered from the Children's Hospital Oakland Research Institute) and cloned into the transformation vectors pWR and pWRh (NHB, unpublished). The region of DNA included in each construct is as follows, with coordinates

relative to base 1 defined as the A of the first *Pax* ATG. Pax: −960 to 6460 and 9083 to 25821 (deleting three *lectin* genes within a *Pax* intron) with GFP inserted between the last codon and the stop codon (25110/1) with four serines as a linker; PDLP: 16754 to 24366; and BAC2: 26373 to 37451. Transgenic flies were generated, and $Pax^{\Delta 1}$/CyO; P{w+, rescue}/+ flies were crossed to each other. The presence of non-CyO $Pax^{\Delta 1}/Pax^{\Delta 1}$ flies were scored for rescue. To confirm rescue, DNA was extracted and analysed by PCR to confirm the presence of the deletion, the absence of the wild type locus, and the presence the GFP tagged *Pax* gene.

To generate $Pax^{\Delta 1}$ deficient germ-line clones, males carrying *hs-flp; FRT40A* ovo^{D1}*/CyO* were crossed to $Pax^{\Delta 1}$ *FRT40A/CyO* females. Larvae from this cross were heat shocked daily for 1 hour at 37°C during 3 days, and the emerging $Pax^{\Delta 1}$ *FRT40A/FRT40A* ovo^{D1} adult females were crossed to $Pax^{\Delta 1}$/CyO, P{wg^{en11} *LacZ*} males.

In situ hybridization and antibody staining

Fluorescent *in situ* hybridization with TSA amplification system (Perkin-Elmer) and immunohistochemistry was done as described previously (Junion et al., 2002). To generate RNA probes for *Mp20* (primers used: 5′-CCAGCAAGCGCAATCCCG-3′ and 5′-GAGGTTCTGGCCAGCCTG-3′) and *Pax* (5′-GACGAAGCAATCCGGATGC-3′ and 5′-CAGCACTCGTCGACAGTGC-3′) the corresponding DNA sequences were cloned by PCR in pGemTeasy. The corresponding antisense RNAs were transcribed *in vitro* using T7 or SP6 RNA polymerase. For *mspo*, Gold collection clone RE52725 was used to generate RNA probe. For fluorescent staining, the following antibodies were used: guinea pig anti-Eve (1:1000; from D. Kosman, University of California, USA), mouse anti-Lbe (1:2500; Jagla et al., 1998), rabbit anti-Slou (1:400; gift of M. Frasch), rabbit anti-β3-Tubulin (1:5000; from R. Renkawitz-Pohl, Philipps Univ., Germany), rabbit anti-Tm2 (1:50; from J. Sparrow, Univ. York, UK), rabbit anti-MHC (1:200; from D. Kiehart, Duke University, USA), rabbit anti-LacZ (1:1000; Sigma), goat anti-GFP (1:300; Biogenesis). Cy3, Cy5 and 488 conjugated secondary antibodies were used (1:300; Jackson Immuno-Research). Embryos were mounted in Fluoromount-G antifade reagent (Southern Biotech). Labeled embryos were analyzed using an LSM510 Meta (Zeiss) confocal microscope.

Staging embryos and nuclei counting

In addition to embryonic morphology, the dorsal closure and gut morphology was used for precise staging all embryos. Staining against the identity genes were used to determine the number of nuclei in specific muscles: Eve in DA1, Lbe in SBM and Slou in DT1, VA2 and VT1 muscles. Nuclei were counted using 40x objective on a Zeiss LSM510 Meta confocal microscope. For each muscle/mutant condition analyzed, 30 (Table S1, S3, S5-S7) or 40 (Table S2) abdominal hemisegments (A2-A4) at stages 15 were counted (minimum 10 embryos). For kinetics analyses, 30 hemisegments were counted at stage 12 (7.20-9.20h AEL), 13 (9.20-10.20 h AEL), 14 (10.20-11.20h AEL) and at early (11.20-12h AEL) and late stage 15 (12-13h AEL) (Table S7). Data plots and statistical analyses were performed with Prism 5.0 using Kruskal-Wallis and Dunns post-tests.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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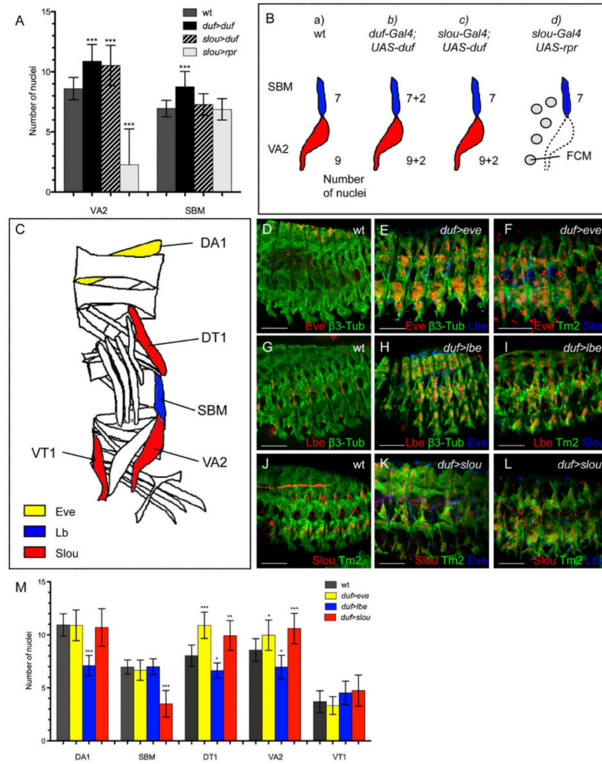

Figure 1. FCs but not FCMs control muscle-type specific fusion programmes

(A) Number of nuclei in SBM and VA2 in *wt*, *duf-Gal4;UAS-duf slou-Gal4;UAS-duf* and *slou-Gal4;UAS-rpr* stage 15 embryos stained for Tm2 and Slou or for ß3-Tub and Lb. The histogram (and all following histograms) show the mean number of nuclei and error bars show standard deviation from the mean. Asterisks show the significance of variation compared to the *wt*. The values used to plot the graphs are shown in Table S2. All driver controls are presented in Figure S1 and Table S1. (M) Schematic representation of fusion control in SBM and VA2. (a) In *wt* condition the SBM has 7 nuclei and the VA2 9 nuclei. (b) Pan-muscular *duf* overexpression leads to an increase in nuclei number in both muscles. (c) Local reduction of FCM number by increasing number of fusion events in the VA2 or (d) local increase of free FCMs by inducing apoptosis in VA2, do not modify the SBM fusion programme.

(C) Schematic representation of Eve, Lbe and Slou expression patterns. (D-M) Modified fusion programmes in embryos with ectopic expression of *eve*, *lbe* and *slou*. (D-L) Stage 15 embryos are stained for ß3-Tub or Tm2 (green) to label all muscles and for Eve (D-F, H, K), Lb (G-I, E, L) or Slou (J-L, F, I) to reveal myoblast nuclei in a subset of muscles. *wt* (D, G, J) and *duf-Gal4;UAS-eve* (E, F), *duf-Gal4;UAS-lbe* (H, I), *duf-Gal4;UAS-slou* (K, L) embryos are shown, dorsal is up and anterior is left. Scale bar correspond to 50µm. In mutant contexts, ectopic expression of Eve, Lbe and Slou is in red and endogenous expression in blue. (M) Number of nuclei in DA1, SBM, DT1, VA2 and VT1 muscles in wild type stage 15 embryos and in embryos with *duf-Gal4*-driven expression of *eve*, *lbe* and *slou* (see Table S3).

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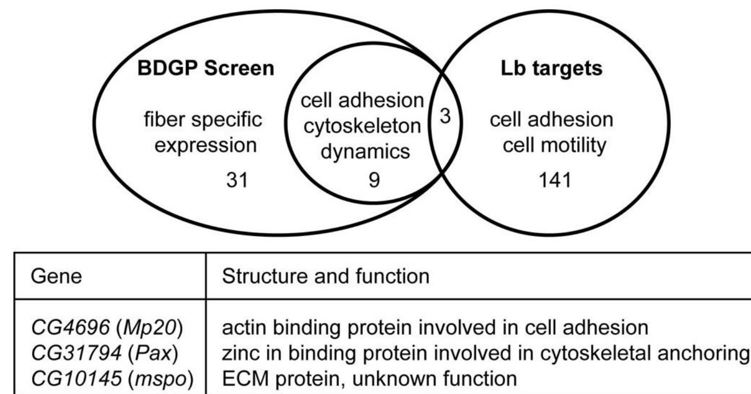

Gene	Structure and function
CG4696 (*Mp20*)	actin binding protein involved in cell adhesion
CG31794 (*Pax*)	zinc in binding protein involved in cytoskeletal anchoring
CG10145 (*mspo*)	ECM protein, unknown function

Figure 2. Selection of candidate genes for muscle-specific regulation of fusion process

To select candidates we compared two pools of genes: i) according to BDGP *in situ* database 554 genes are expressed in larval/embryonic muscle system, among them, 31 show a potential muscle-specific expression (Table S4) and 9 fit into GO category “cell adhesion” or “cytoskeleton dynamics”; ii) The second pool of 141 genes corresponds to a subset of *lb* target genes (Junion et al., 2007) belonging to the category “cell adhesion/cell motility”. Comparison between these two screens allows us to identify 3 candidates.

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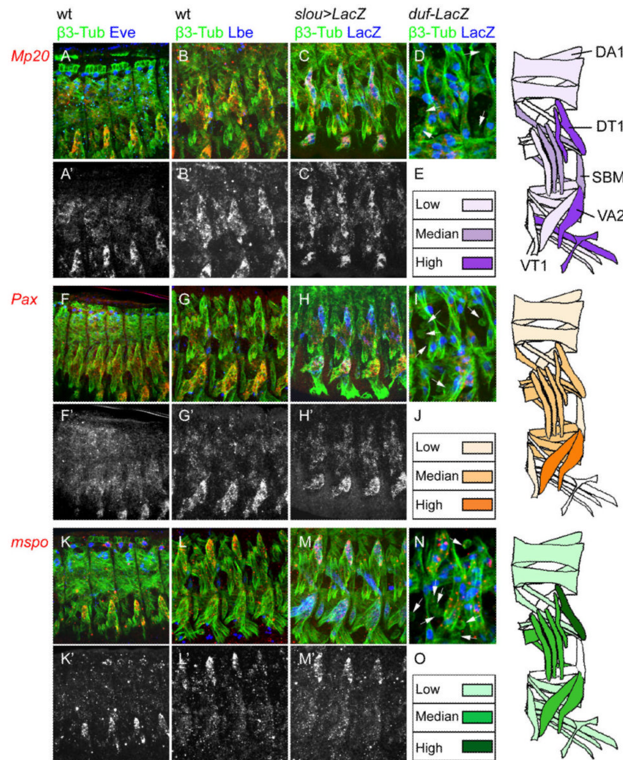

Figure 3. *Mp20*, *Pax* and *mspo* display muscle-type specific expression levels

Expression patterns of *Mp20* (A-E), *Pax* (F-J) and *mspo* (K-O). (A-C', F-H', K-M') *in situ* hybridization to reveal *Mp20*, *Pax* or *mspo* transcripts (red) coupled to ß3-Tub (green) and muscle-specific staining (blue) for Eve in the DA1 (A, F, K), for Lbe in the SBM (B, G, L) in *wt* embryos, or for LacZ (C, H, M) in *slou-Gal4;UAS-LacZ* context to visualize DT1, LO1, VA2 and VT1 muscles. Lateral views of three abdominal segments from stage 15 embryos are shown. Panels (A'-C', F'-H', K'-M') correspond to the red channel only. (D, I, N) *Mp20, Pax* and *mspo* expression (in red) in stage 14 *duf-LacZ* embryos. Growing muscles are visualized with ß3-Tub (green) and LacZ (blue) staining. During fusion process, *Mp20, Pax* and *mspo* are expressed only in growing muscles and not in unfused FCMs (white arrows). (E, J, O) Schematic representation of *Mp20* (E), *Pax* (J) and *mspo* (O) expression levels. High, median and low expression levels are represented by the colour intensity.

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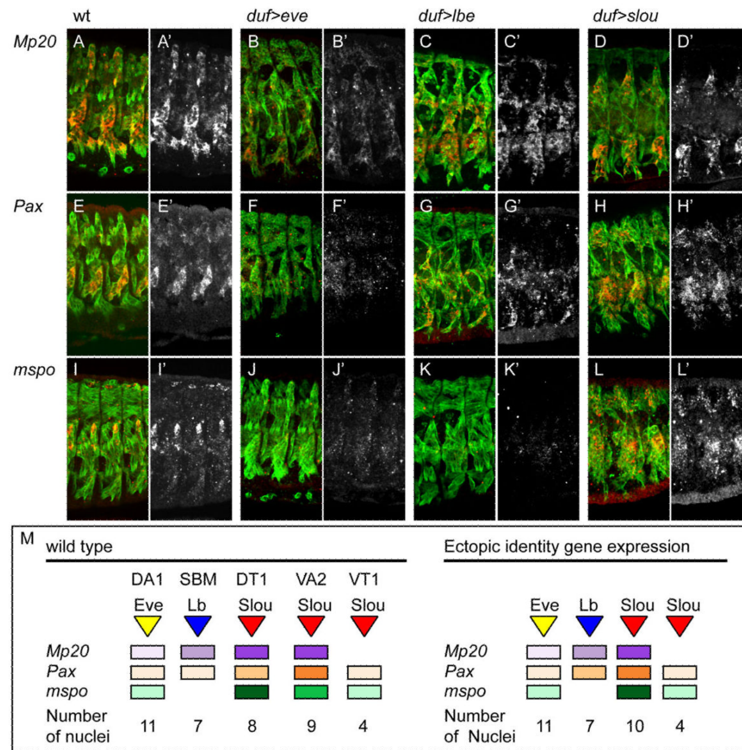

Figure 4. Identity genes regulate *Mp20, Pax* and *mspo* expression levels

Mp20 (A-D), *Pax* (E-H) and *mspo* (I-L) expression patterns in wt (A, E, I), *duf-Gal4;UAS-eve* (B, F, J), *duf-Gal4;UAS-lbe* (C, G, K) and *duf-Gal4;UAS-slou* (D, H, L). For each condition, three abdominal segments of stage 15 embryos are shown. *Mp20, Pax* or *mspo* transcripts (red) are revealed by *in situ* hybridization coupled to staining for ß3-Tub (green). Panels (A'-L') show the red channel only. Complementary dorsal views are presented in Figure S3.

(M) Schematic representation of identity genes dependent regulation of *Mp20, Pax* and *mspo* expressions and corresponding fusion programmes in DA1, SBM, DT1, VA2 and VT1 muscles. In *wt* embryos, Eve, Lb and Slou induce a specific expression level of *Mp20, Pax* and *mspo* leading to the execution of a specific fusion programme. Ectopic Eve, Lbe and Slou modulate target's expression level and leads to the induction of a new fusion programme mimicking that induced by a given identity gene in *wt* condition.

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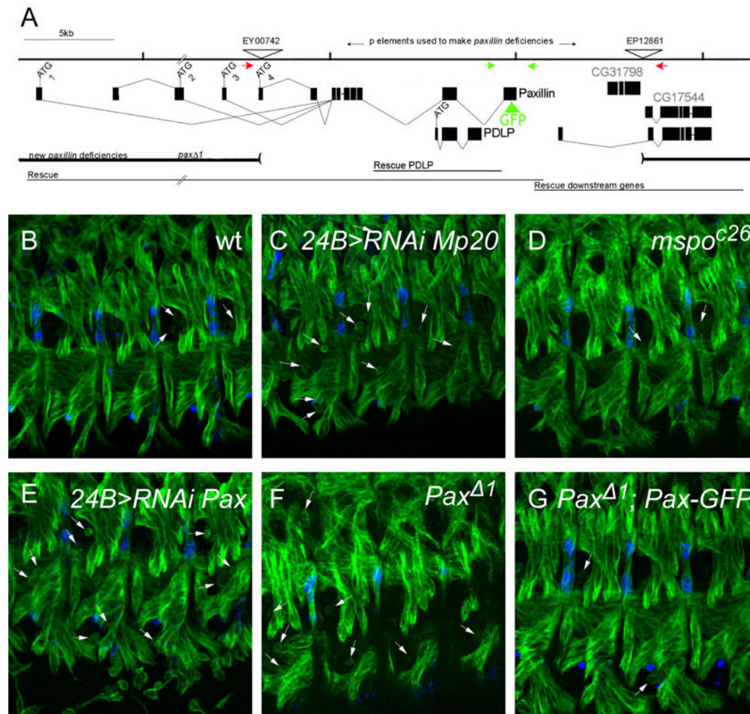

Figure 5. Loss of *Mp20* or *Pax* functions leads to discrete fusion defects

(A) Diagram of the *Pax* locus, showing the four *Pax* transcripts and the short *Pax*-derived LIM only protein (PDLP). Two P-element insertions, EY00742 and EP12861, were used to generate deletions of the locus. Rescue constructs encompassing the *Pax* locus, *PDLP* only, or the downstream genes were generated. Red arrows indicate the position of the primers used to screen the candidate deletions. Green arrows represent the primers used to differentiate the wt allele *vs* the rescued *Pax* allele.

(B) *wt* and (C-G) loss of function contexts for *Mp20, Pax* and *mspo*. Lateral views of stage 15 embryos stained for β3-Tub (green) and Lbe (blue) are shown. Arrows indicate unfused FCMs. Compared to the *wt* (A) an increased number of unfused cells is present in *24B-Gal4; UAS*-RNAi *Mp20* (C), *24B-Gal4; UAS*-RNAi *Pax* (E) and *Pax*$^{\Delta 1}$ (F) embryos. In *mspo* mutant embryos (D) and in *Pax*$^{\Delta 1}$*; Pax-GFP* rescue context (G) the number of unfused cells is similar to that in the *wt*.

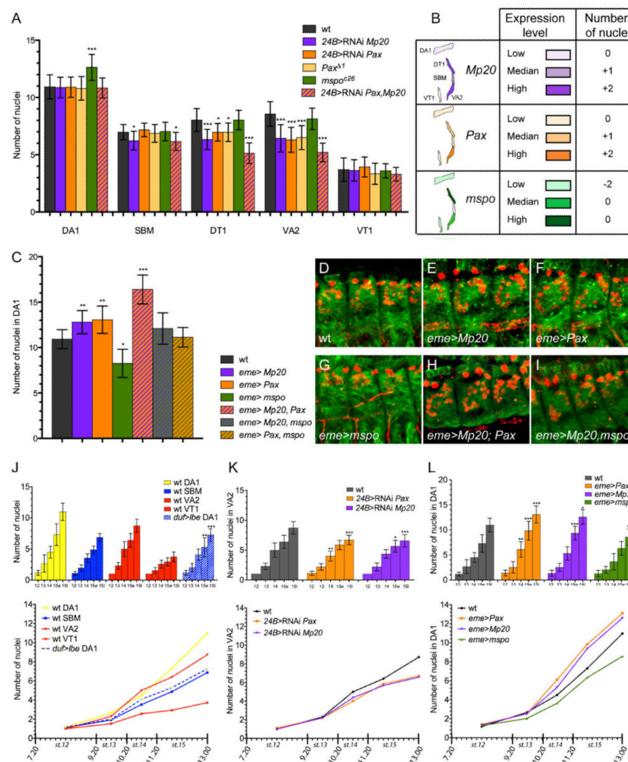

Figure 6. *Mp20, Pax* and *mspo* modulate fusion process in a muscle-type specific manner and according to their expression level

(A) Number of nuclei present in DA1, SBM, DT1, VA2 and VT1 muscles at stage 15 embryos analyzed in wt, *24B-Gal4; UAS*-RNAi *Mp20, 24B-Gal4; UAS*-RNAi *Pax*, $Pax^{\Delta 1}$, $mspo^{c26}$ and *24B-Gal4; UAS*-RNAi *Pax; UAS*-RNAi *Mp20*. To determine the number of nuclei, embryos were double-stained for Eve (DA1), Lbe (SBM) or Slou (DT1, VA2, VT1) and for β3-Tub or Tm2. Bar graphs show the mean number of nuclei and asterisks indicate the significance of variation compared to the wt. The mean values used to plot the graphs are shown in Table S5. (B) Summary of *Mp20*, *Pax* and *mspo* functions correlated to their expression levels.

(C-I) Effect of *Mp20*, *Pax* and *mspo* gain of function on number of nuclei in DA1 muscle. (C) Bar graphs show the mean number of nuclei in DA1 muscle, in wt and in gof conditions for *Mp20*, *Pax* and *mspo* and asterisks indicate the significance of variation compared to the wt. Values used to plot the graphs are shown in Table S6.

(D-I) Stage 15 embryos stained for Eve and β3-Tub. Dorsal portion of three segments in wt, eme-Gal4;UAS-Mp20, eme-Gal4;UAS-Pax, eme-Gal4;UAS-mspo and double gof contexts *eme-Gal4;UAS-Pax;UAS-Mp20* and *eme-Gal4;UAS-Mp20;UAS-mspo* are shown.

(J-L) Kinetics of fusion in wt and *duf-Gal4; UAS-lbe* (J) in *Pax* and *Mp20* attenuation (K) and *Pax, Mp20* and *mspo* overexpression (L) contexts. VA2 and DA1 muscles were analysed in attenuation and overexpresion contexts, respectively. In upper panels bar graphs show the mean number of nuclei, asterisks show the significance of variation compared to the wt. The values used to plot the graphs are shown in Table S7. In lower panel, only mean values are used to visualize the number of nuclei according to time AEL.

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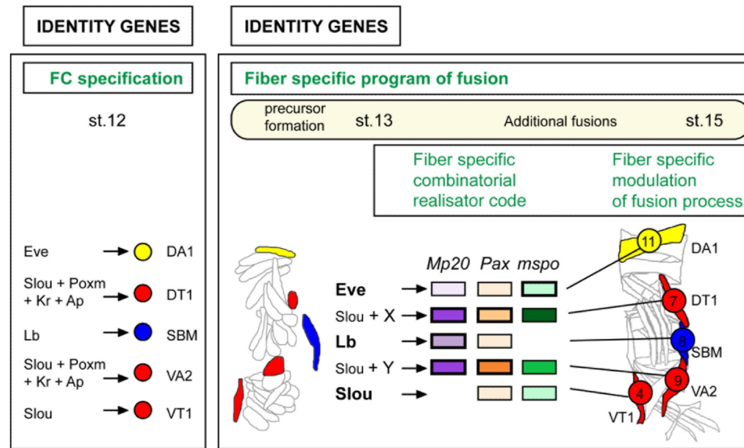

Figure 7. Identity genes control muscle specific fusion programmes by determining combinatorial code of expression levels of their targets

The identity genes *eve, lb* and *slou* are required for specification of FCs that give rise to DA1, DT1, SBM, VA2 and VT1 muscles. The FC specification step is completed by stage 12 of embryogenesis but expression of *eve*, *lb* and *slou* continues in later stages. Between, stage 12 and 15, FCs fuse with a determined number of FCMs to generate muscles with a specific number of nuclei. *eve, lb* and *slou* induce the recruitment of 10, 6 and 3 nuclei respectively, leading to the DA1, SBM and VT1 formation. Slou in combination with unidentified factors induce two other fusion programmes for DT1 and VA2 muscles. To execute muscle-specific fusion programmes, the identity genes act *via Mp20, Pax* and *mspo* by establishing a combinatorial code of target's expressions. Bold outlines show the target activity in fusion programme.