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The conserved transcription factor Mef2 has multiple roles in adult *Drosophila* musculature formation

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SUMMARY

Muscle is an established paradigm for analysing the cell differentiation programs that underpin the production of specialised tissues during development. These programs are controlled by key transcription factors, and a well-studied regulator of muscle gene expression is the conserved transcription factor Mef2. In vivo, Mef2 is essential for the development of the *Drosophila* larval musculature: *Mef2*-null embryos have no differentiated somatic muscle. By contrast, a similar phenotype has not been seen in analyses of the function of *Mef2* genes in other examples of myogenesis. These include using conditional mutant mice, using morpholinos in zebrafish and using hypomorphic mutants in *Drosophila* adult development. However, we show here that *Mef2* is absolutely required for a diverse range of *Drosophila* adult muscle types. These include the dorso-longitudinal muscles (DLMs), the largest flight muscles, which are produced by tissue remodelling. Furthermore, we demonstrate that *Mef2* has temporally separable functions in this remodelling and in muscle maintenance. *Drosophila* adult muscles are multi-fibre and physiologically diverse, in common with vertebrate skeletal muscles, but in contrast to *Drosophila* larval muscles. These results therefore establish the importance of *Mef2* in multiple roles in examples of myogenesis that have parallels in vertebrates and are distinct from that occurring in *Drosophila* embryogenesis.

KEY WORDS: Mef2, Myogenesis, Remodelling, Muscle differentiation, *Drosophila*

INTRODUCTION

Cell differentiation programs underpin the production of specialised tissues during development and are controlled by key transcription factors. Muscle is both one of the main paradigms for analysing cell differentiation programs and a major tissue. In muscle, the conserved transcription factor Mef2 has a prominent position for two main reasons. First, studies in mammalian cell culture showed that Mef2 factors cooperate with the MyoD family in the regulation of muscle gene expression and in myogenic activity (Black and Olson, 1998). Second, a role in muscle differentiation in vivo was shown in *Drosophila* through analysing its single *Mef2* gene in larval musculature development during embryogenesis (Lilly et al., 1995; Bour et al., 1995; Ranganayakulu et al., 1995). Most strikingly, *Mef2*-null mutants have no differentiated somatic muscle, the equivalent to vertebrate skeletal muscle, whereas different *Mef2* hypomorphic mutants have different amounts of residual muscle. This absolute requirement for *Mef2* in muscle differentiation led to it being used as an example for understanding how transcription factors control complex cell differentiation programs (Junion et al., 2005; Sandmann et al., 2006; Elgar et al., 2008).

Drosophila has two phases of myogenesis: the first produces the larval musculature during embryogenesis; the second produces the adult musculature during metamorphosis. In common with vertebrate skeletal muscles, but in contrast to those in *Drosophila* larvae, adult *Drosophila* muscles are multi-fibre, and physiologically and functionally distinct (Bate, 1993; Bernstein et al., 1993; Taylor, 2006; Maqbool and Jagla, 2007). In the adult thorax, the largest muscles are the jump muscle or TDT, the leg muscles and the indirect flight muscles, which comprise two groups – the dorso-longitudinal muscles (DLMs) and the dorso-ventral muscles (DVMs) (Miller, 1950). The DVMs, TDT and leg muscles are formed de novo during metamorphosis from adult myoblasts associated with the imaginal discs. By contrast, the DLMs form through tissue remodelling (Fernandes et al., 1991).

In adult *Drosophila*, the first *Mef2* hypomorphic mutants tested had a reduced number of abnormally patterned DLMs (Ranganayakulu et al., 1995; Cripps and Olson, 1998; Nguyen et al., 2002). More recently, a study using a temperature-sensitive combination of *Mef2* alleles found that, under restrictive conditions, the DLMs, DVMs and TDT still form, although again the number of DLMs was reduced (Baker et al., 2005). It was concluded that adult myogenesis can proceed independently of *Mef2*. In vertebrates, *Mef2* functional analysis in vivo is complicated by the presence of multiple *Mef2* genes (a-d). However, a conditional knockout revealed that *Mef2c* functions in sarcomere assembly, although other aspects of muscle differentiation still occurred (Potthoff et al., 2007). Morpholinos against *Mef2c* plus *Mef2d* produced a related phenotype in zebrafish (Hinits and Hughes, 2007). Together, these results question whether the absolute requirement of *Mef2* for myogenesis is restricted to *Drosophila* embryonic development.

Previously, have found that the *Him* gene can result in no DLMs at the end of pupation (Soler and Taylor, 2009). As *Him* encodes an inhibitor of Mef2 activity (Liotta et al., 2007), this suggested that DLM development may in fact require *Mef2*. Here, we tested this directly and analysed the wider role of *Mef2* in adult *Drosophila* myogenesis by using RNAi to produce *Mef2* loss of function. Genetic analysis of adult *Drosophila* development has been transformed by the application of genome-wide transgenic RNAi libraries (Dietzl et al., 2007). This approach is effective in muscle and is especially useful for genes with earlier functions in

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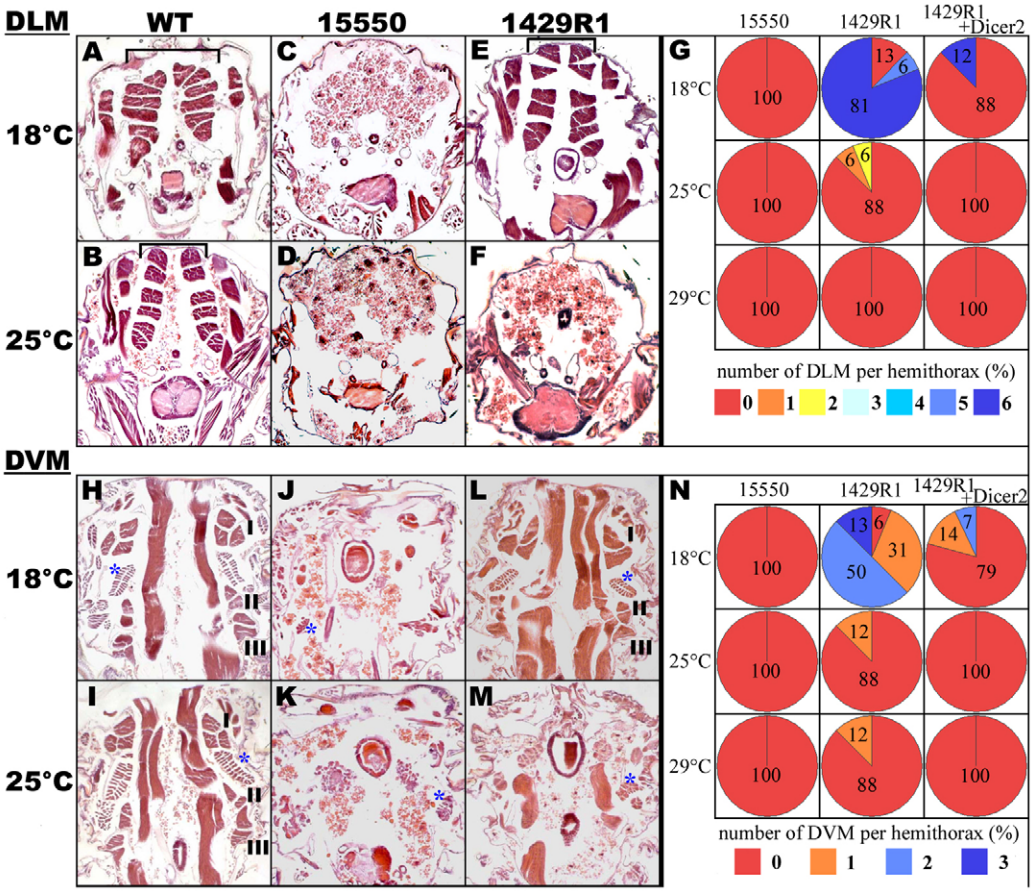

Fig. 1. *Mef2* is required for *Drosophila* adult fibrillar muscles. Wax sections at 96h APF to visualise the DLMs and DVMs in wild type (WT), and *1151Gal4>UASMef2RNAi(15550)* or *UASMef2RNAi(1429R-1)* at 18°C and 25°C. (**A-F**) DLMs (square bracket). Dorsal is uppermost. (**H-M**) DVMs. The three groups of DVM fibres are indicated (I, II, III) as is the TDT (asterisk). Anterior uppermost. (**G**,**N**) Quantitative analysis of the effect of *1151Gal4>UASMef2RNAi(15550)* or *UASMef2RNAi(1429R-1)* and *1151Gal4;;UASDicer2>UASMef2RNAi(1429R-1)* at 18°C, 25°C and 29°C on DLM number (G) and DVM number (N). Percentages for each muscle number are shown. At least 32 hemithoraces were analysed for each condition. Images for 29°C are in supplementary material Fig. S1.

embryogenesis (Schnorrer et al., 2010). We used this tool to control the place, extent and timing of gene function knockdown and show that adult *Drosophila* myogenesis cannot proceed independently of *Mef2*. Rather, it is absolutely required for a diverse range of muscle types. These include the DLMs for which we show *Mef2* is required for both their remodelling and to prevent their degeneration.

MATERIALS AND METHODS

Drosophila stocks, crosses and sample preparation

The following Drosophila stocks were used: *1151-Gal4* (Anant et al., 1998), *1151-Gal4;Mhc-tauGFP* (Soler et al., 2004), $Mef2^{65}$*/CyOtwiGal4UAS-2xEGFP*, $Mef2^{113}$*/CyOtwiGal4UAS-2xEGFP* (Elgar et al., 2008), $Mef2^{30\text{-}5}$*/CyOKrGFP*, $Mef2^{44\text{-}5}$*/CyOKrGFP* (Baker et al., 2005), $TubGal80^{ts}$, *UASRab-RP4DN* (Bloomington stock centre), *UASRab-RP4RNAi* and *UASDicer2* (Dietzl et al., 2007). The UASMef2RNAi lines were 15550 and 15549 (Dietzl et al., 2007), and 1429R-1 and 1429R-3 (NIG). 1151-Gal4;Mhc-tauGFP was the control and the driver for all experiments except those with $Gal80^{ts}$, which used 1151-Gal4. Egg collection and culture was at 25°C (unless otherwise stated). All pupal ages are hours after pupa formation (APF) equivalent to the time of development at 25°C.

1151Gal4;;UASMef2RNAi/$TubGal80^{ts}$ eggs were collected at 25°C and transferred to a permissive temperature, 19°C, until L2. Larvae were then shifted to 30°C, a restrictive temperature for $Gal80^{ts}$, until 13.5h APF (equivalent to 20h APF at 25°C) and then 'down-shifted' to the permissive temperature (19°C). $Mef2^{30\text{-}5}$/$Mef2^{44\text{-}5}$ progeny were selected from a cross of $Mef2^{30\text{-}5}$*/CyOKrGFP* × $Mef2^{44\text{-}5}$*/CyOKrGFP* and switched to the restrictive temperature as described (Baker et al., 2005). $Mef2^{65}$/$Mef2^{113}$ progeny were selected from a cross of $Mef2^{65}$*/CyOtwiGal4UAS-2xEGFP* × $Mef2^{113}$*/CyOtwiGal4UAS-2xEGFP*.

Tissue preparation and microscopic analysis for thoracic and leg muscles were essentially as previously described (Soler et al., 2004; Soler and Taylor, 2009).

RESULTS AND DISCUSSION

Mef2 is required for adult fibrillar muscles

We used *1151-Gal4* to drive expression of *UASMef2RNAi(15550)* in the adult myoblasts of second instar larvae and then continuing throughout adult myogenesis (Anant et al., 1998; Soler et al., 2004). Normally, there are six DLMs in each hemithorax at the end of pupal life. By contrast, this RNAi-induced *Mef2* loss-of-function resulted in the complete absence of DLMs at 96h APF (Fig. 1A-D). An independent *UASMef2RNAi* construct (1429R-1) also had this effect (Fig. 1E,F,G). At 18°C there were six DLMs in ~80% of examples, but at increased temperature there were again no DLMs; in nearly 90% of examples at 25°C, and in 100% at 29°C. This is consistent with the temperature-dependent activity of the Gal4/UAS system, which results in higher levels of UAS gene expression at higher temperature (Brand et al., 1994). We assume that the 1429R-1 line expresses *Mef2RNAi* at a lower level than the 15550 line. Provision of Dicer2, a component of the RNAi

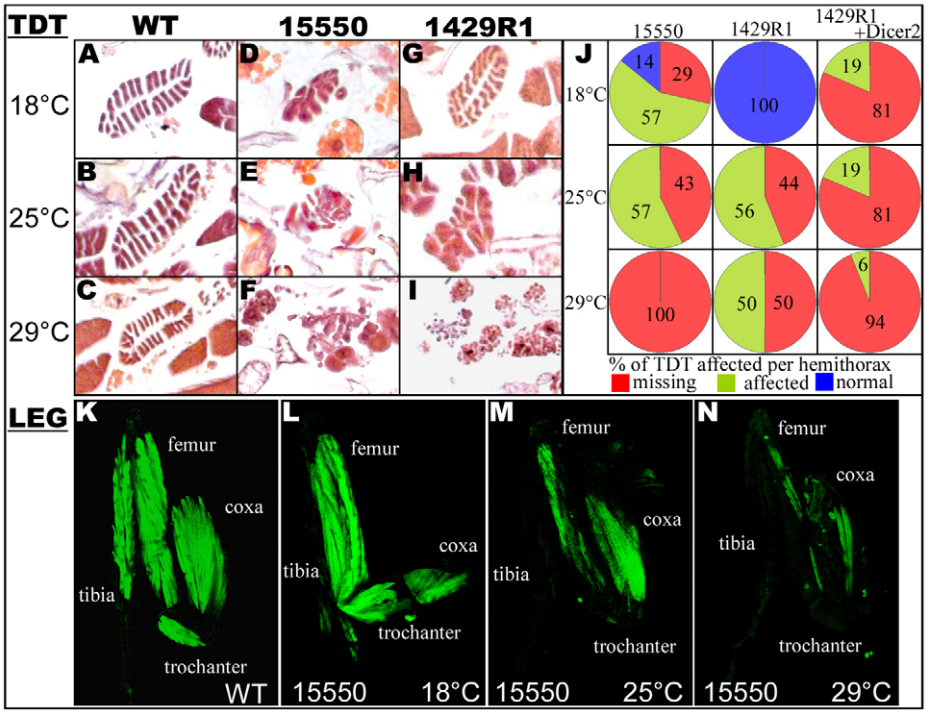

Fig. 2. *Mef2* is required for *Drosophila* adult tubular muscles. (**A-I**) Wax sections at 96h APF to visualise the TDT in wild type (WT), and *1151Gal4>UASMef2RNAi(15550)* or *UASMef2RNAi(1429R-1)* at 18°C, 25°C and 29°C. (**J**) Quantitative analysis of the effect of *1151Gal4>UASMef2RNAi(15550)* or *UASMef2RNAi(1429R-1)* and *1151Gal4;;UASDicer2>UASMef2RNAi(1429R-1)*. At least 32 hemithoraces were analysed for each condition. (**K-N**) Confocal micrographs visualizing the leg muscles using Mhc-GFP in wild type and *1151Gal4>UASMef2RNAi(15550)* at 18°C, 25°C and 29°C. *UASMef2RNAi(1429R-1)* gave a similar, but weaker, phenotype (supplementary material Fig. S2).

machinery that can enhance the transgenic RNAi effect (Dietzl et al., 2007), also enhances the phenotype so that at 18°C there were no DLMs in nearly all cases (Fig. 1G).

We found that *Mef2* is also required for the DVMs, the second major group of fibrillar thoracic muscles, which develop de novo, rather than by remodelling like the DLMs (Fig. 1H-N). Either *Mef2RNAi* 15550 or 1429R-1 can result in the complete absence of these muscles at the end of pupation. As with the DLMs, the 1429R-1 line required a higher temperature, or Dicer2, to give this strong phenotype.

Mef2 is required for adult tubular muscles

A structurally and physiologically distinct thoracic muscle is the jump muscle or TDT (Peckham et al., 1990). It is a tubular muscle with fibres organised around a central lumen. We found it also requires *Mef2* (Fig. 2A-J). Its organisation is affected in the weaker *Mef2RNAi*-induced *Mef2* loss-of-function conditions (Fig. 2D,E,H,J), but with the 15550 line at 29°C it was absent in all cases. We then investigated whether *Mef2* is required for the development of other tubular muscles, those of the leg, where pairs of multi-fibre muscles are organised around a central tendon (Soler et al., 2004). We monitored leg myogenesis using MhcGFP (Soler et al., 2004) and found it was affected by *Mef2* loss-of-function similarly to the TDT (Fig. 2K-N). Thus, with the 15550 line at 18°C there was some inhibition and this was greater at 25°C. At 29°C there was very little properly differentiated muscle. In most instances there was no MhcGFP in the tibia and in only a few fibres more proximally; in some there was no MhcGFP at all.

In summary, and in contrast to a previous conclusion (Baker et al., 2005), we found that *Mef2* is absolutely required for a diverse range of muscles. Significantly, these comprise structurally distinct muscles, both fibrillar and tubular in organisation, muscles that develop in radically different ways, through either remodelling or de novo assembly, and muscles with physiologically distinct properties. Furthermore, we note that tubular muscle may require less *Mef2* activity than does fibrillar muscle. This is suggested by the presence of both the TDT and some leg muscles under conditions in which the DLMs and DVMs are absent, e.g. with *Mef2RNAi(15550)* at 25°C, and could be linked to the different genetic programs in fibrillar and tubular muscle (Schonbauer et al., 2011).

Muscle remodelling requires *Mef2*

DLM development begins shortly after pupal formation with remodelling of three muscles, the larval oblique muscles, in each hemithorax. These muscles resist the general histolysis of metamorphosis, then fuse with adult myoblasts and split to form the six DLM templates. In wild-type myogenesis, this splitting is described as starting at 14h APF and being complete by 20h APF (Fernandes et al., 1991). To assess thoroughly the effect of *Mef2* on this remodelling, we assayed the number of developing DLM fibres in serial wax sections at three time-points (22h, 26h and 30h APF). This approach demonstrated that muscle remodelling requires *Mef2* (Fig. 3). With *Mef2RNAi(15550)* at 25°C limited splitting produced averages of 3.6 fibres at 22h APF and 3.5 fibres at 26h APF, in contrast to the six in the control (Fig. 3J). In the stronger loss-of-function conditions of *Mef2RNAi(15550)* plus Dicer2 at 29°C, splitting was almost completely inhibited (3.3 fibres at 26h APF, not shown).

This requirement for *Mef2* is consistent with the only previous observation of a *Mef2* phenotype during splitting in which the average DLM number at 23h in the $Mef2^{65}/Mef2^{113}$ heteroallelic combination was approximately 3.7 (Cripps and Olson, 1998). However, only this single time-point was assessed during remodelling and so one could not exclude a delay in splitting, which we have seen under some *Mef2* loss-of-function conditions. We found an average of 4.3 fibres by 26h in $Mef2^{65}/Mef2^{113}$ (Fig. 4).

A temporally separable function of *Mef2* in muscle maintenance

Our findings suggest there may be temporally distinct functions for *Mef2* in adult myogenesis. We tested this by controlling expression of the *UASMef2RNAi* transgene using $Gal80^{ts}$, a temperature-

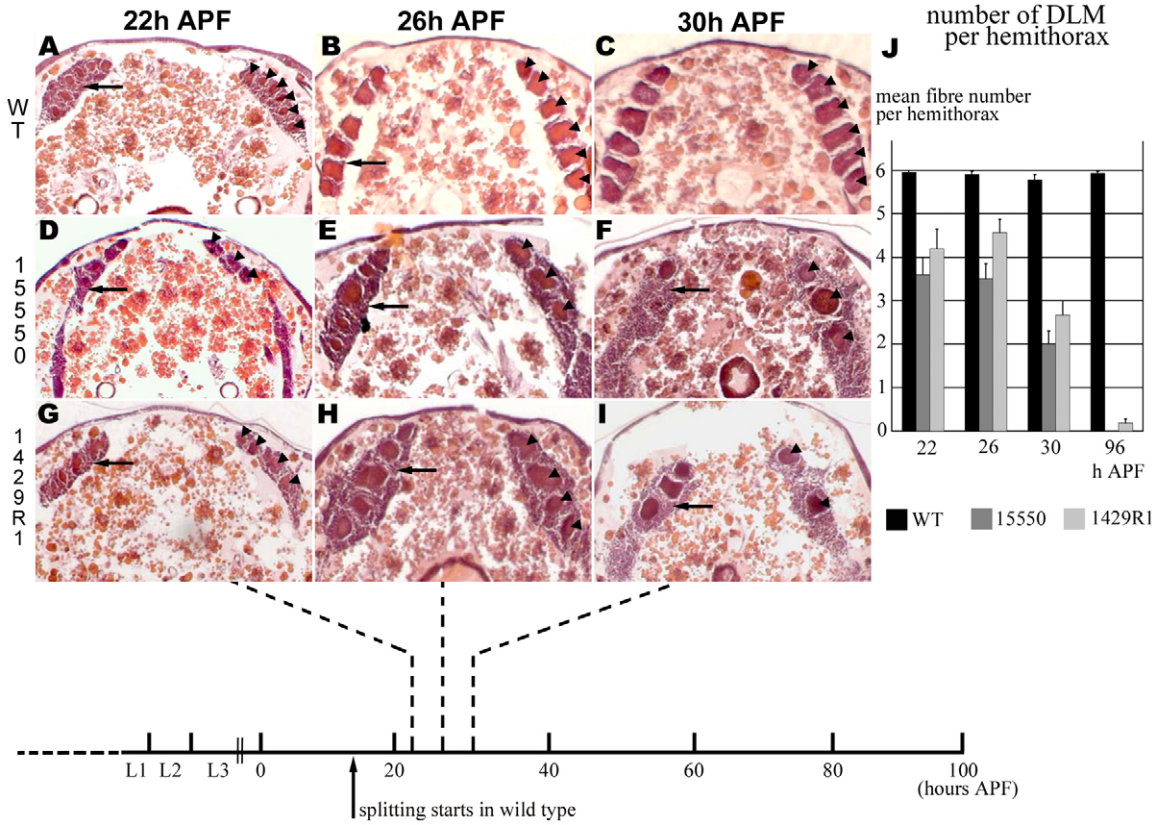

Fig. 3. Muscle remodelling requires *Mef2*. (**A-I**) Wax sections at three time-points during remodelling (22h, 26h and 30h APF) above a timeline of development in wild type (WT), and *1151Gal4>UASMef2RNAi(15550)* or *UASMef2RNAi(1429R-1)* at 25°C. The effect of *1429R-1* was again less strong than *15550*. Arrowheads indicate muscle fibres; arrows indicate unfused myoblasts. (**J**) Histogram showing the mean fibre number and s.e.m. for each condition (at least 10 hemithoraces analysed for each).

sensitive form of the Gal4-inhibitor Gal80 (McGuire et al., 2003; supplementary material Fig. S3). We undertook a 'down-shift' experiment. This produced *Mef2* loss of function during early adult myogenesis using a restrictive temperature for $Gal80^{ts}$ and restored function later at a permissive temperature. In this regime with *Mef2RNAi(15550)*, remodelling was inhibited with an average of 3.6 fibres at 26h APF, but rather than there being no muscles at 96h APF, there was an average of 2.2 (Fig. 4A-C). With the 1429R-1 line, averages were 4.3 and 2.2 fibres, respectively (Fig. 4C). Thus, restoring *Mef2* function during myogenesis indicates a temporally separable function for *Mef2* after remodelling in promoting muscle maintenance. This echoes the role of *Mef2* in regulating the expression of genes at different stages in the *Drosophila* embryonic muscle development program (Sandmann et al., 2006; Elgar et al., 2008).

The idea of temporally separable functions of *Mef2* is supported by our experiments without $Gal80^{ts}$ that show that *Mef2* is required to prevent the degeneration of split, as well as un-split, DLM templates. Thus, in the milder *Mef2* loss-of-function conditions of *Mef2RNAi(1429R-1)* at 25°C, full splitting as indicated by six fibres at 26h APF is seen in 39% of cases (Fig. 3J and data not shown). However, these remodelled fibres then degenerate, as indicated by 88% of cases having no DLMs at 96h APF, and none having more than two (Fig. 1G). DLM fibre loss starts soon after splitting as the fibre number is already decreased by 30h APF (Fig. 3J). This is consistent with our previous findings with overexpression of the Mef2-inhibitor Him, which showed DLM degeneration under way at 30h APF (Soler and Taylor, 2009). Remodelling and maintenance are important aspects of tissue biology. The functions for *Mef2* that we demonstrated in them may have implications for muscle repair.

Mef2 point mutants and *Mef2* RNAi form an allelic series

Previous observations of the first hypomorphic *Mef2* mutants tested and then on other alleles, including a temperature-sensitive combination, found a reduced number of DLMs at the end of pupation (Ranganayakulu et al., 1995; Cripps and Olson, 1998; Nguyen et al., 2002; Baker et al., 2005). Here, we confirmed that the average DLM number at 96h APF in both the previously described $Mef2^{65}/Mef2^{113}$ and temperature-sensitive $Mef2^{30\text{-}5}/Mef2^{44\text{-}5}$ heteroallelic combinations was approximately three (Fig. 4C). However, in both there were examples with more than three DLMs, implying that at least some template splitting occurs, and other examples with fewer than three DLMs, indicating that some muscle degeneration occurs.

To compare systematically the phenotype of these *Mef2* loss-of-function conditions to our *Mef2RNAi* analysis, we monitored the DLMs throughout their development at 22h, 26h, 30h and 96h APF. We found that, in both $Mef2^{65}/Mef2^{113}$ and $Mef2^{30\text{-}5}/Mef2^{44\text{-}5}$, although template splitting is reduced, some does indeed occur. At 26h APF, the average DLM fibre number was 4.3 for $Mef2^{65}/Mef2^{113}$ and 4.0 for $Mef2^{30\text{-}5}/Mef2^{44\text{-}5}$ (Fig. 4C). This, together with our findings with *Mef2RNAi* and previously with the

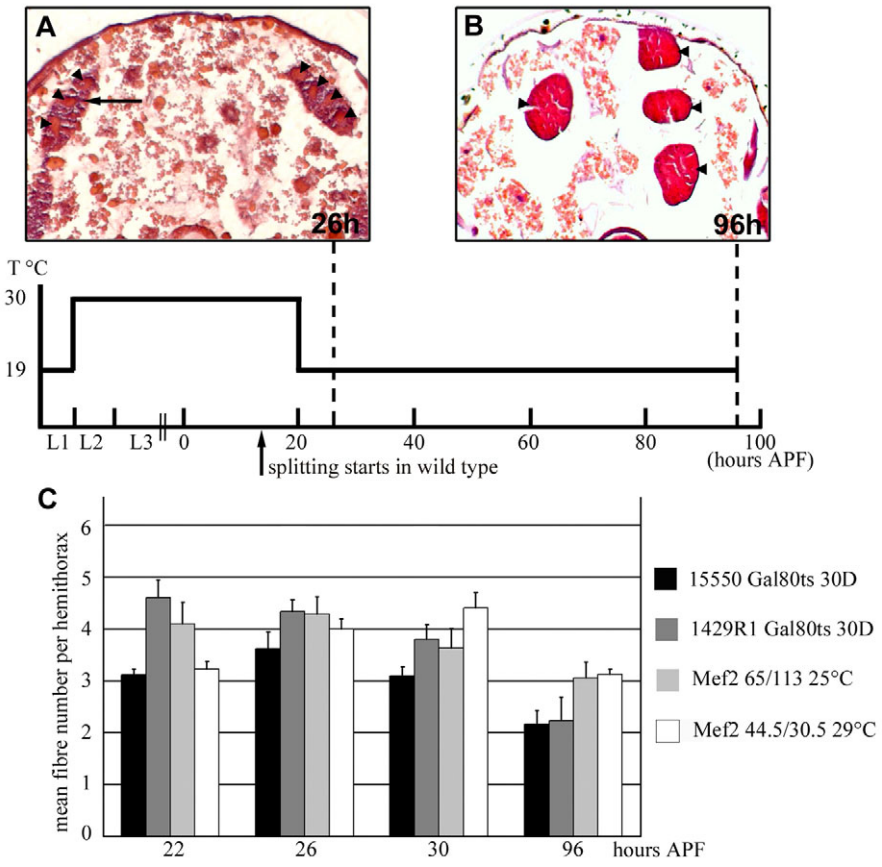

Fig. 4. *Mef2 RNAi* demonstrates temporally separable functions for *Mef2* and forms an allelic series with *Mef2* point mutation alleles. (**A**,**B**) Wax sections at two time-points (26h and 96h APF) above a timeline of development showing the temperature shifts used to control *Mef2RNAi* expression in *1151Gal4;;UASMef2RNAi(15550)/TubGal80^{ts}* larvae/pupae. Arrowheads indicate muscle fibres; arrows indicate unfused myoblasts. (**C**) Histogram comparing the mean fibre number and s.e.m. for 'down-shift' experiments (30D) using *Mef2RNAi* (15550 or 1429R-1) with *Mef2^{65}/Mef2^{113}* and *Mef2$^{30-5}$/Mef2$^{44-5}$* (at least eight hemithoraces analysed for each). Images for *Mef2^{65}/Mef2^{113}* and *Mef2$^{30-5}$/Mef2$^{44-5}$* are in supplementary material Fig. S4.

Mef2-inhibitor Him (Soler and Taylor, 2009), shows that the DLM number at the end of pupation depends on the extent of both remodelling and maintenance. Not separating effects on these two phenomena can confound analyses. For example, the route to three DLMs at 96h APF need not be no splitting followed by complete muscle maintenance.

Our quantitative assessment shows that the various *Mef2* loss-of-function conditions all inhibit splitting (Figs 3, 4) and can be placed in the following series: *Mef2RNAi(15550)>Mef2^{65}/Mef2^{113}*, *Mef2$^{30-5}$/Mef2$^{44-5}$>Mef2RNAi(1429R1)*. However, the effect on the final DLM number is strikingly different. Whereas both RNAi conditions result in zero DLMs, the two heteroallelic combinations each results in approximately three DLMs. Significantly our 'down-shift' experiment demonstrates that *Mef2RNAi* can give a phenotype that resembles the heteroallelic combinations (Fig. 4). Splitting is substantially inhibited and muscles are maintained. We conclude that the various *Mef2RNAi* conditions and the existing *Mef2* mutants together comprise an allelic series and that the strong *Mef2* loss-of-function DLM phenotype is both an inhibition of remodelling and an absence of DLMs at the end of pupation. We rationalise previous work (Baker et al., 2005) by suggesting that in the *Mef2* heteroallellic combinations there is Mef2 function that can efficiently activate muscle maintenance genes, but not those required for splitting. Differential gene activation in these alleles has been noted (Baker et al., 2005; Lovato et al., 2009).

Despite the prominence of Mef2 among the transcription factors that regulate muscle gene expression (Black and Olson, 1998; Sandmann et al., 2006), muscle development during *Drosophila* embryogenesis has previously stood out as the only demonstration of *Mef2* loss of function that results in no muscle differentiation in vivo. Therefore, our findings considerably extend the roles of *Mef2* and do so into types of myogenesis that have many parallels with vertebrate muscle development. It remains to be determined whether further similarities with respect to *Mef2* function will be uncovered in vertebrates through earlier and/or more complete knock-down of all relevant *Mef2* family members. Our results can be viewed alongside another study that was 'in press' when this paper was submitted (Bryantsev et al., 2012). It also found that one of the *Mef2RNAi* lines we used resulted in a lack of adult somatic muscles. Additionally, it reported unfused myoblasts around the DLMs and concluded that myoblast fusion requires *Mef2*. We too observed these unfused myoblasts (Fig. 3F,I). A role for *Mef2* during fusion is consistent with our previous finding that the Mef2 inhibitor Him is downregulated just prior to fusion (Soler and Taylor, 2009).

As documented in the *Drosophila* embryo (Gunthorpe et al., 1999; Elgar et al., 2008), it may be that levels of Mef2 activity also matter during the diverse events of adult myogenesis. Thus, there were conditions that had little effect on DLM remodelling but that resulted in their degeneration, that resulted in no fibrillar muscles while tubular muscles remained, or that affected distal leg muscles more than proximal. These effects, together with the range and temporal separation of processes affected, all highlight the multiple inputs of transcription factors such as Mef2 that need to be understood to appreciate how complex cell differentiation programs are orchestrated.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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