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...
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<sup>65/CyOtwiGal4UAS-2xEGFP, Mef2<sup>113/CyOtwiGal4UAS-2xEGFP</sup></sup> (Elgar et al., 2008), Mef2<sup>30-5/CyOKrGFP, Mef2<sup>44-5/CyOKrGFP</sup></sup> (Baker et al., 2005), TubGal80ts, 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 Gal80ts, 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/TubGal80ts 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 Gal80ts, until 13.5h APF (equivalent to 20h APF at 25°C) and then ‘down-shifted’ to the permissive temperature (19°C). Mef2<sup>65/65</sup> progeny were selected from a cross of Mef2<sup>65/CyOtwiGal4UAS-2xEGFP × Mef2<sup>113/113</sup>CyOtwiGal4UAS-2xEGFP</sup></sup>. 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
Furthermore, we note that tubular muscle may require less novo assembly, and muscles with physiologically distinct properties develop in radically different ways, through either remodelling or de muscles, both fibrillar and tubular in organisation, muscles that of muscles. Significantly, these comprise structurally distinct Mef2 (2005), we found that 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 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 (Femandes 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<sup>65</sup>/Mef2<sup>213</sup> 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<sup>65</sup>/Mef2<sup>213</sup> (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<sup>ts</sup>, a temperature-
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 Gal80ts 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 Gal80ts 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, 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).

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 Mef265/Mef2113 and Mef265/Mef244-5, although template splitting is reduced, some does indeed occur. At 26h APF, the average DLM fibre number was 4.3 for Mef265/Mef2113 and 4.0 for Mef265/Mef244-5 (Fig. 4C). This, together with our findings with Mef2RNAi and previously with the
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) > Mef265/Mef2113, Mef230-5/Mef244-5 > 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 heteroallelic 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.

Acknowledgements
We are grateful to Richard Cripps, the VDRC and the NIG-Fly and Bloomington Stock Centres for Drosophila stocks; to Anne Franke and Rebecca Callard for assistance in some experiments; and to Cardiff School of Biosciences Histology Unit.
References


