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Differential requirements for Myocyte Enhancer Factor-2 during adult myogenesis in Drosophila

Anton L. Bryantsev, Phillip W. Baker, TyAnna L. Lovato, MaryAnn S. Jaramillo, Richard M. Cripps *

Department of Biology, University of New Mexico, Albuquerque, NM 87131, USA

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ABSTRACT

Identifying the genetic program that leads to formation of functionally and morphologically distinct muscle fibers is one of the major challenges in developmental biology. In Drosophila, the Myocyte Enhancer Factor-2 (MEF2) transcription factor is important for all types of embryonic muscle differentiation. In this study we investigated the role of MEF2 at different stages of adult skeletal muscle formation, where a diverse group of specialized muscles arises. Through stage- and tissue-specific expression of *Mef2* RNAi constructs, we demonstrate that MEF2 is critical at the early stages of adult myoblast fusion: mutant myoblasts are attracted normally to their founder cell targets, but are unable to fuse to form myotubes. Interestingly, ablation of *Mef2* expression at later stages of development showed MEF2 to be more dispensable for structural gene expression: after myoblast fusion, *Mef2* knockdown did not interrupt expression of major structural gene transcripts, and myofibrils were formed. However, the MEF2-depleted fibers showed impaired integrity and a lack of fibrillar organization. When *Mef2* RNAi was induced in muscles following eclosion, we found no adverse effects of attenuating *Mef2* function. We conclude that in the context of adult myogenesis, MEF2 remains an essential factor, participating in control of myoblast fusion, and myofibrillogenesis in developing myotubes. However, MEF2 does not show a major requirement in the maintenance of muscle structural gene expression. Our findings point to the importance of a diversity of regulatory factors that are required for the formation and function of the distinct muscle fibers found in animals.

Introduction

Studying myogenesis in model organisms provides insights into the genetic causes of human muscular diseases, as divergent species are thought to utilize similar strategies in muscle development and maintenance. *Drosophila melanogaster* has long been used as a tool in dissecting genetic and molecular mechanisms of muscle development.

Development of somatic muscles in *Drosophila* occurs at two stages of the life cycle. First, during the embryonic phase, all muscle types arise from the mesoderm via an intense burst of cell specification and tissue-type differentiation. Fusion of numerous fusion-competent myoblasts to individual founder cells creates multi-nucleate myofibers that elongate and adhere to designated cuticular attachment sites (Baylies and Michelson, 2001; Beckett and Baylies, 2006; Dohrmann et al., 1990). Following fusion, nascent myofibers activate the expression of muscle structural genes, and the larval somatic muscles activate a relatively uniform set of these genes: the muscles invariably express the embryonic muscle actin gene, *Act57B* (Kelly et al., 2002); as well as the troponin C gene *TpnC73F* (with other troponin C gene products being non-detectable by hybridization *in situ* (Herranz et al., 2004)), and other muscle structural genes (Arredondo et al., 2001; Gasch et al., 1988; Zhang and Bernstein, 2001). Hence, at the end of embryonic myogenesis, somatic muscles appear as arrays of individual myofibers, arranged in a largely consistent pattern in each body segment, and sharing a relatively uniform expression of muscle structural genes.

There is compelling evidence that the MADS domain transcription factor Myocyte Enhancer Factor-2 (MEF2) plays an essential role at the embryonic stage of muscle development. Although specification of muscle precursors proceeds normally in a *Mef2* mutant background, these mutants show a profound lack of multinucleate myotubes (Bour et al., 1995; Lilly et al., 1995; Paululat et al., 1999; Ranganayakulu et al., 1995). Consistent with this observation, many structural muscle genes have functional MEF2-binding sites in their enhancers (Kelly et al., 2002; Lin et al., 1996; Sandmann et al., 2006; Tanaka et al., 2008). *Drosophila* MEF2 is a transcriptional activator, capable of initiating expression of these target genes autonomously, even in foreign environments such as the embryonic ectoderm or S2 cells in tissue culture (Lin et al., 1997a; Tanaka et al., 2008).

At the second phase of *Drosophila* myogenesis, that occurs during pupal development, pre-existing larval muscles become histolyzed and adult muscles develop *de novo*. Adult myofibers arise from adult muscle precursor myoblasts, that have been preserved throughout the larval stage as small clusters of cells associated with the nerves and imaginal discs (Dutta et al., 2004; Rivlin et al., 2000). During metamorphosis,

* Corresponding author.
E-mail address: rcripps@unm.edu (R.M. Cripps).

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adult muscle precursors proliferate and migrate toward fusion sites (Currie and Bate, 1991; Fernandes et al., 1991; Roy and VijayRaghavan, 1997), where myoblast fusion is initiated by sparse founder cells that play essentially the same roles as in embryogenesis (Dutta et al., 2004; Rivlin et al., 2000). In one specific case, being the formation of the adult dorsal longitudinal indirect flight muscles, the role of founder cells is taken by a subset of persistent larval myofibers (Fernandes et al., 1991). Following fusion, newly-developed myofibers enter the hypertrophic phase of growth, where the muscle volume increases due to massive expression of structural genes and assembly of the contractile apparatus.

While superficially the process of adult skeletal myogenesis appears analogous to muscle development in the embryo, muscle formation in adults results in significantly more diverse groups of myofibers, due to the appearance of new specialized muscles. Specialized somatic muscles in adult flies include the indirect flight muscles (IFMs), the tergal depressor of the trochanter (TDT, or jump muscle), direct flight muscles at the base of the wing, as well as head and leg muscles (Bernstein et al., 1993). Muscles in adults that resemble the relatively uniform larval muscle type are restricted to the abdominal body wall (Baker et al., 2005; Currie and Bate, 1991). Clearly, adult myogenesis in *Drosophila* shows both similarities and differences with embryonic muscle development: specification of founder cells, myoblast fusion, and muscle differentiation are common processes; on the other hand, adult muscles are highly divergent from embryonic muscles in size, arrangement, ultrastructure, and physiology (reviewed in Bernstein et al., 1993).

The broad spectrum of specialized adult muscles correlates with the distinct and specific functions that they perform. For example, the most prominent thoracic muscles, the TDTs and IFMs, generate power for jumping and flying, respectively, and their different behavioral functions are correlated with these muscles having different morphological characteristics (see Peckham et al., 1990). Based upon their ultrastructure, the IFMs belong to the fibrillar type of muscles, whereas the TDTs are of a tubular type, reflecting the architecture and organization of their myofibrils. These muscles are different at the molecular level, too: IFMs express the muscle-specific actin gene *Act88F,* and the troponin C gene *TpnC41F*; while TDTs express *Act79B* and *TpnC41C* (Fyrberg et al., 1983; Herranz et al., 2004). The *Act57B* and *TpnC73F* genes, expressed in all embryonic and larval skeletal muscles, show strong restriction in expression at the adult stage, to the abdominal body wall muscles (Baker et al., 2005; Herranz et al., 2004). Nevertheless, some muscle-specific genes retain their persistent expression in all adult muscles, including the *Myosin heavy chain* (*Mhc*) gene (Hess et al., 2007). Altogether, the understanding of how transcriptional regulation is controlled in somatic muscles during the transition from larval to adult musculature – allowing activation of some new genes and shutting down other ones, while keeping some genes active all along – has become a major research question.

The role of MEF2 in adult myogenesis remains obscure. Our laboratory previously assessed the role of MEF2 in adult myogenesis using *Mef2* temperature-sensitive alleles (Baker et al., 2005). The results of our study revealed that *Mef2* down-regulation caused relatively mild defects in adult muscle formation, in remarkable contrast to the severe muscle defects observed in embryos under similar experimental conditions. The weak adult phenotype was also demonstrated in earlier studies that employed different *Mef2* hypomorphic mutants (Nguyen et al., 2002; Ranganayakulu et al., 1995). Thus, it was suggested that the requirement for MEF2 function in adult myogenesis is somewhat reduced. However, it was still clear that both the hypomorphic and temperature-sensitive mutants retained some small MEF2 function, and we specifically raised the possibility that low levels of residual MEF2 function were still sufficient to support myogenesis. It remained to be determined how a more exacerbated effect upon MEF2 activity might impact adult myogenesis.

In this study, we have taken an RNAi approach to knockdown *Mef2* function in the developing adult muscles, at various steps of adult myogenesis. We find that silencing of *Mef2* in adult myoblasts leads to a massive loss of somatic muscles, due to inability of the myoblasts to fuse, to activate expression of muscle genes, and to generate myofibers. By contrast, silencing of *Mef2* in post-fusion myofibers has less dramatic effects on fibrillogenesis and expression of structural muscle genes. Finally, silencing of *Mef2* in post-eclosion muscles does not produce a detectable deleterious effect on muscular structure and performance during adult life. We conclude that, in adult muscles, MEF2 remains a critical factor for myoblast fusion and initiation of muscle structural gene expression. However, MEF2 becomes less essential for the maintenance of structural gene expression, and its role is taken by other factors. Our study provides new data for understanding the developmental program necessary for formation of divergent, specialized groups of muscles such as those that are found in higher animals.

Materials and methods

Molecular cloning

The cloning of the *Mef2* inverted repeat (IR) construct was done following the strategy described before (Bao and Cagan, 2006). An approximately 700-bp region of the *Mef2* coding sequence, present in all annotated *Mef2* transcripts (Fig. 1A), was amplified using the forward 5′-ACTCTAGACCACCATTTGTCCATTAAGCA and the reverse 5′-GTTCTAGACTGGAGTGGGTGTGATGTGG primers. The underlined regions in the primer sequences are non-genomic sequence, added to achieve cleavage of the PCR product with *Xba*I. Amplicons digested with *Xba*I and thereby containing the CTAG overhangs at each end, were cloned into the vector pGEM-WIZ (obtained from the Drosophila Genomic Resource Center (DGRC), IN, USA) via the end-compatible *Avr*II restriction site. The orientation of the insert was verified by analytical restriction digest. In the second round of cloning, the same *Xba*I-treated amplicons were inserted into the pGEM-WIZ already containing one sense-oriented amplicon, via the *Xba*I restriction site, and the resulting clones were screened for those bearing tail-to-tail amplicon insertions, separated by a short intron provided by the vector. Selected clones were verified by direct sequencing. Next, the whole construct, containing the two amplicons separated by the intron, was cut out of the pGEM-WIZ with *Spe*I and inserted into pUAST (Brand and Perrimon, 1993) via compatible ends produced by digesting pUAST with *Xba*I. The final construct, pUAST-C–Mef2–IR(2), or IR2, was verified by sequencing, and contained the amplified *Mef2* part oriented in the sense direction, followed by the intron, and its own antisense-oriented copy.

Fragments of *Act79B* and *Act88F* upstream sequences were amplified by PCR and cloned into the pCaSpeR-hs-AUG-ßGal (CHAB) vector (Thummel and Pirrotta, 1992). For generation of Gal4 drivers, the enhancer sequences recapitulating the entire expression patterns of *Act88F* and *Act79B* genes were first amplified via PCR. The primers used in these reactions were forward 5′-GAAGAGCATTGGCACCAA and reverse 5′-TGACAATAGGCTCTCCGTTT to amplify the *Act79B* enhancer; and forward 5′-TTGCACTGATAAATGGTCGG and reverse 5′-CGGACCTTAGAAGGACCGA to amplify the *Act88F* enhancer. Both amplicons were then cloned into the vector pChsGal4 (Apitz, 2002). The orientation and integrity of the inserts were verified by direct sequencing of the final clones.

Cell culture co-transfection assay

In order to test the silencing potency of the IR2 construct, S2 cells were co-transfected with: pPacPl–Gal4, to activate expression from the IR2 construct; pUAST-C–Mef2–IR(2), the *Mef2* silencing construct; pPacPl–Mef2(wt) (Tanaka et al., 2008), to express MEF2; and the reporter pC9-CHAB, bearing the MEF2-responsive enhancer of *Act57B* fused to *LacZ* (Kelly et al., 2002). The base-line expression control