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A Temporal Map of Transcription Factor Activity: Mef2 Directly Regulates Target Genes at All Stages of Muscle Development

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Summary

Dissecting components of key transcriptional networks is essential for understanding complex developmental processes and phenotypes. Genetic studies have highlighted the role of members of the Mef2 family of transcription factors as essential regulators in myogenesis from flies to man. To understand how these transcription factors control diverse processes in muscle development, we have combined chromatin immunoprecipitation analysis with gene expression profiling to obtain a temporal map of Mef2 activity during *Drosophila* embryonic development. This global approach revealed three temporal patterns of Mef2 enhancer binding, providing a glimpse of dynamic enhancer use within the context of a developing embryo. Our results provide mechanistic insight into the regulation of Mef2's activity at the level of DNA binding and suggest cooperativity with the bHLH protein Twist. The number and diversity of new direct target genes indicates a much broader role for Mef2, at all stages of myogenesis, than previously anticipated.

Introduction

Embryogenesis requires the establishment of complex spatiotemporal patterns of gene expression, which are tightly orchestrated to ensure proper progression through development. High-throughput in situ hybridization screens have dramatically increased our knowledge of the number of genes expressed at a specific time and cell type during development. Yet, information on how these precise patterns are established, maintained, and dissipated remains very limited. Understanding the regulation of these complex patterns will require dissecting the components of key transcriptional networks, identifying the location of active cis-regulatory modules (CRMs), and understanding the regulatory code within them.

Many key transcriptional regulators involved in muscle development have been identified in a wide range of species. Genetic studies highlighted the essential role of the myocyte enhancing factor 2 (Mef2) and the myogenic regulatory factor (MRF) families of transcription factors. Nevertheless, due to the small number of characterized muscle enhancers, our understanding of the transcriptional combinatorial code required to regulate expression in different myoblast subpopulations is very limited. The temporal regulation of coordinated gene expression, necessary for an ordered progression through muscle development, has only been addressed in a limited number of recent studies (Gaudet et al., 2004; Penn et al., 2004).

Understanding Mef2 function during vertebrate development is complicated by potential functional redundancy between the four family members, which are expressed in overlapping patterns during embryogenesis. The genome of *Drosophila melanogaster* contains only one *Mef2* gene, which, like in vertebrates, is expressed in all muscle types (Nguyen et al., 1994). This provides a unique opportunity to dissect the regulatory role of myocyte enhancing factors in vivo. The DNA binding domain of Mef2 proteins is highly conserved, as is their recognition of a degenerate consensus (Black and Olson, 1998) and their essential function in muscle development (Bour et al., 1995; Lin et al., 1997b; Naya et al., 2002; Wang et al., 2003). This suggests that Mef2 regulation of muscle genes in *Drosophila* is likely to be evolutionary conserved. For many of the Mef2 target genes identified to date, this is certainly the case (reviewed by Black and Olson, 1998).

Previous studies aimed at identifying genes directly regulated by Mef2 fall into two broad categories: computational studies based on the prediction of Mef2 binding sites, or experimental identification of Mef2 bound promoter regions.

Computational prediction of sites bound by Mef2 is complicated by the degeneracy of its consensus binding site. Additional constraints, limiting the search space to regions close to known muscle genes or assuming clustering with additional motifs, for example, can reduce the number of false positive predictions. However, this precludes the discovery of enhancer regions not conforming to the formulated model. A recent study in *Drosophila* (Junion et al., 2005) verified 62 out of 99 computational predictions of Mef2 bound enhancers by using chromatin immunoprecipitations and provided the largest number of Mef2-regulated modules to date. Although this prediction-based approach identified new targets with high accuracy, due to the restrictions used in the model only a limited fraction of the genome was sampled and only known mesodermal genes could be identified as targets.

Two studies applied an experimental approach, chromatin immunoprecipitation of Mef2 from mammalian C2C12 cells, to identify promoters bound by Mef2A (Paris et al., 2004) or Mef2C (Blais et al., 2005). While more than 20 target genes were identified in each study, the analysis of enriched sequences with promoter or CpG island arrays could only detect Mef2 binding close to transcriptional start sites, a very limited fraction of the genome. Additionally, though C2C12 cells are a well-established model by which to study differentiation, the extent to which this system mimics muscle development in vivo remains unclear.

Therefore, the studies conducted to date cannot provide an unbiased map of Mef2 enhancer binding during development. The extent to which this transcription

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factor regulates the myogenic program remains unclear, as an accurate estimate of the number of enhancers bound in vivo and of the regulated target genes is not available. Moreover, the dynamics of Mef2 activity during development have not been addressed to date.

We have used an unbiased approach to identify Mef2 bound enhancers and Mef2 direct target genes during multiple consecutive stages of the developing *Drosophila* embryo. Complementary genomic approaches were integrated to systematically understand the function of this important transcription factor: (1) chromatin immunoprecipitation followed by microarray analysis (ChIP-on-chip) with genomic tiling arrays was used to map the position of Mef2 bound enhancers; (2) gene expression profiling of *Mef2* mutant embryos during a time course of development revealed genes requiring Mef2 for their correct expression.

The combination of ChIP-on-chip (distinguishing between direct and indirect regulation) and gene expression profiling of a loss-of-function mutant identified more than 200 direct target genes, many of which are differentially regulated in *Mef2* mutants. Analyzing multiple time points of development identified three temporal profiles of enhancer occupancy, revealing regulation of Mef2's activity at the level of DNA binding. The diverse functions of Mef2 direct target genes highlight a much broader role for Mef2 in myogenesis than previously anticipated and place Mef2 at the center of the myogenic transcriptional hierarchy.

Results and Discussion

Enhancer Occupancy during Embryonic Development

To identify enhancer regions bound by Mef2 in vivo, chromatin immunoprecipitation followed by microarray analysis (ChIP-on-chip) was performed at five consecutive time points of embryogenesis spanning key stages of muscle development (Figure 1A; Table S1; see the Supplemental Data available with this article online). To systematically identify Mef2 bound genomic regions in an unbiased manner, we constructed a *Drosophila* genomic tiling array, taking advantage of genomic clones generated by the Berkeley *Drosophila* Genome Project (BDGP) to sequence the *Drosophila* genome (Adams et al., 2000). The array consists of overlapping 3 kb fragments tiling across ~50% of the *Drosophila* genome.

For each developmental time period assayed, four independent embryo collections, chromatin preparations, and chromatin immunoprecipitations were performed (Figure 1B). To avoid false positives due to unspecific binding of a single antibody, we performed the assays with two different polyclonal antisera raised against Mef2. Only genomic regions that were significantly enriched by both anti-Mef2 antibodies, but not in mock experiments, were considered. This method is likely to be very stringent and therefore provides a high confidence that the enriched regions are specifically bound by Mef2.

These experiments identified 1015 significantly enriched Mef2 bound fragments, with less than 1% estimated false positives. Due to the overlapping nature of the array, this represents 670 nonoverlapping genomic regions bound by Mef2 at one or more developmental time points. To assess the quality of this data set, we determined if regions previously reported to be bound by Mef2 were recovered. Eight of the previously characterized Mef2 direct target genes are covered by our arrays. We identified Mef2 binding in the proximity of all eight genes assayed (Figures 1C and 1D; Figure S1): *Actin 57B* (Kelly et al., 2002), *Muscle LIM protein at 84B* and *Muscle LIM protein at 60A* (Stronach et al., 1999), *β-tubulin60D* (Damm et al., 1998), *Tropomyosin I* (Lin and Storti, 1997), *inflated* (Ranganayakulu et al., 1995), *mir-1* (Sokol and Ambros, 2005), and *Mef2* itself (Cripps et al., 2004). In many cases, our study not only identified the previously reported Mef2 bound enhancer, but also identified additional ones (Figures 1C and 1D, asterisk; Figure S1A).

Five genes are known to be genetically downstream of *Mef2*, though the mechanism of regulation remains unclear: *Myosin heavy chain* (Bour et al., 1995; Lilly et al., 1995), *meso18E* (Taylor, 2000), *muscleblind* (Artero et al., 1998), *nautilus* (Lin et al., 1997a), and *Chorion factor 2* (Bagni et al., 2002). Our results show Mef2 binding to genomic regions close to four of these genes (*Mhc*, *mbl*, *nau*, and *meso18E*) and to a genomic region further 5′ of *CF2* (Figure S1). This indicates that these genes are directly regulated by Mef2 and identifies the location of at least one of their enhancer regions. In summary, the successful identification of Mef2 binding in the vicinity of all known and suspected target genes suggests a high accuracy of our approach. In addition to the 8 known enhancers, we have identified over 650 new Mef2 bound regions.

A High-Resolution Developmental Time Course of Mef2-Dependent Gene Expression

As a complementary approach to assess the molecular function of Mef2, we determined which genes depend on Mef2 activity for their correct expression during embryonic development. The gene expression profile of wild-type embryos was compared to that of stage-matched *Mef2* mutant embryos throughout a developmental time series. Pure populations of *Mef2* homozygous mutant embryos were isolated as described previously (Furlong et al., 2001a, 2001b). For each developmental time point assayed, four independent replicates were analyzed on microarrays containing at least one probe for every predicted gene in the genome.

We examined the expression profiles of *Mef2* mutant embryos at 11 consecutive 1 hr time points of embryogenesis, spanning from 5 to 16 hr of development (stages 9–16; Table S1). An additional time point was added at 18–19 hr, stage 17, to identify genes that are expressed in differentiated muscle. This allowed us to generate a high-resolution map of *Mef2*-dependent temporal changes in gene expression, spanning the stages of mesoderm subdivision, myoblast specification, myoblast fusion, and the initiation of terminal muscle differentiation (Figure 1A).

This study identified 700 genes with significant changes in gene expression in *Mef2* mutant embryos at 2 or more consecutive time points (Figure S2; Table S2). The eight known Mef2 protein-coding target genes are among them, with *Act57B*, *Mlp84B*, *Mlp60A*, and *TmI* showing a greater than 4-fold decrease in expression at multiple stages of development. The differentially expressed genes are significantly enriched in genes expressed in muscle ($p < 0.005$), indicating that many of the

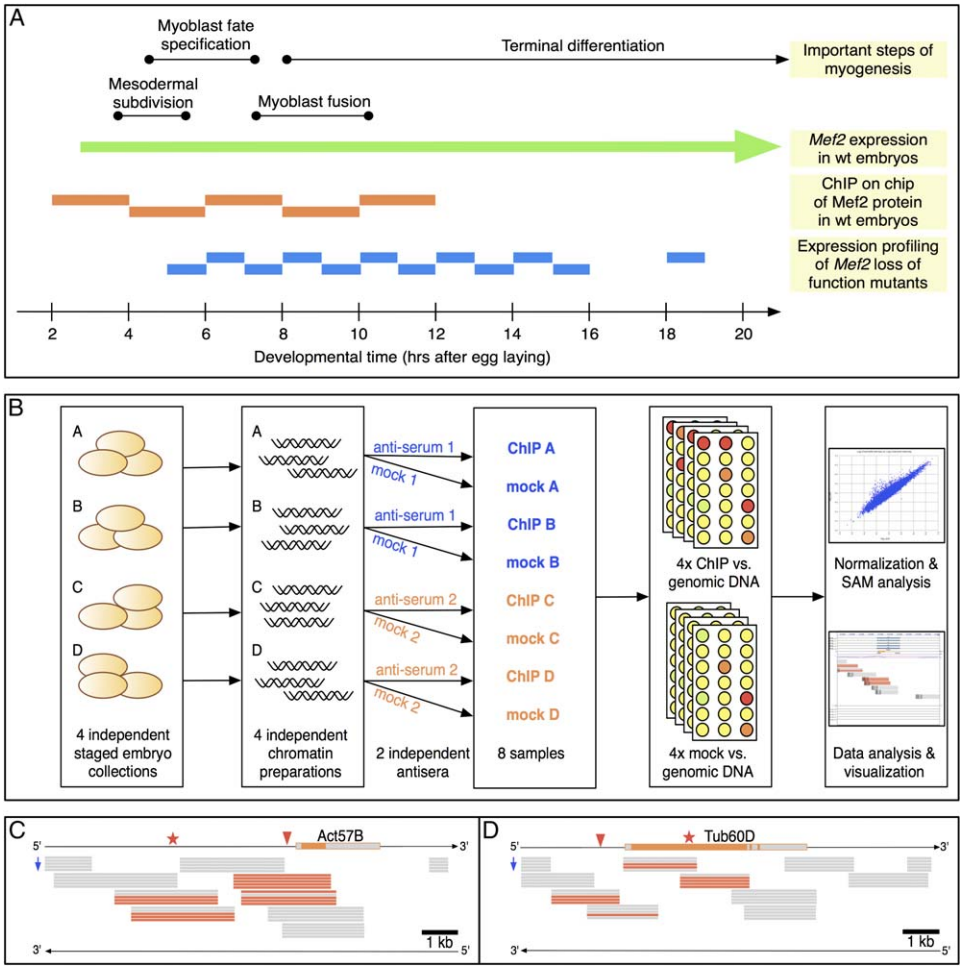

Figure 1. A Global View of Mef2 Binding and Expression Profiling during *Drosophila* Development

(A) Temporal analysis of Mef2 function during myogenesis: Mef2 enhancer binding was assayed at five consecutive 2 hr time points (orange bars), covering the stages of myoblast specification, fusion, and the initiation of terminal differentiation. This information was complemented by expression profiling of *Mef2* mutant embryos at 12 one hr time points (blue bars).

(B) Experimental design of the ChIP-on-chip experiment: for each developmental time point, the four replicate samples were divided into two groups, which were probed with α-Mef2 antisera (ChIP) raised in two different animals and with respective preimmune sera (mock). All eight precipitations were hybridized against a genomic reference DNA sample on genomic tiling arrays. SAM analysis was used to identify regions specifically enriched in ChIP, but not mock, pull-downs.

(C and D) Schematic overviews of two known Mef2 enhancers: the gene loci for *act57B* and *β3Tub60D* are located on the sense strand, indicated at the top of the panels (orange). Genomic fragments on the tiling arrays are indicated as stacks of five horizontal bars in their corresponding genomic position. Each single bar represents the results from one ChIP-on-chip time point, with the earliest (2–4 hr) positioned at the top and the latest (10–12 hr) at the bottom (blue arrow). Significant binding of Mef2, indicated in red, is found for the previously characterized Mef2 binding site (arrowhead) as well as for a previously unknown Mef2 bound region (asterisk).

misregulated genes are expressed in the same cells as *Mef2*.

The function of a substantial number of the differentially expressed genes is unknown. This study indicates that they are genetically downstream of *Mef2* as either direct or indirect targets, and provides a useful resource to identify genes likely to be involved in muscle development. The combination of our ChIP-on-chip results with this expression profiling data allowed us to determine which genes are directly regulated by Mef2.

Identifying Mef2 Direct Target Genes: Integrating ChIP-on-Chip with Gene Expression Data

Genomic tiling arrays provide an unbiased method to identify new regulatory regions independent of their distance to the gene. While this offers a great advantage over promoter arrays, it raises a new challenge for ChIP-on-chip studies: how to accurately match transcription factor bound regions to their correct target genes. Metazoan enhancers have been identified at large distances from their target genes, including within introns of neighboring loci. Assuming that the enhancer is regulating the closest proximal gene will, especially in gene-dense regions, often cause the wrong target gene to be selected.

We have used different sources of metadata to systematically link ChIP-enriched regions to their target genes (Figure S3). The genes in the vicinity of each Mef2 bound region received a cumulative score based on: (1) the distance between a gene and a Mef2 bound

region, (2) a change in expression in *Mef2* mutant embryos, and (3) supporting information, for example about the gene's expression patterns (BDGP in situ database, Flybase, literature). Genes were not assigned based on proximity alone. Using this approach, we identified 211 Mef2 direct target genes with high confidence (Table S3), including all known targets covered by the tiling array.

To estimate the accuracy of our automated gene assignment, we used a collection of characterized enhancers from single gene studies (Bergman et al., 2005; Gallo et al., 2006). A total of 33 of the Mef2 bound regions assigned to target genes with a high confidence score overlap with a previously identified gene's enhancer. In 28 cases (84.8%), the regulated locus was correctly identified (Table S4), illustrating the accuracy of our gene assignment strategy. The remaining five sequences map to the *Enhancer of split* region and were assigned to a different member of this gene cluster. A total of 29 additional Mef2 bound regions overlapped with known enhancer regions. The 12 regulated target genes were not assigned by using our automated approach, as no additional supporting evidence was available. Combining the automatic assignments with information about known regulatory relationships yielded 234 unique genes directly regulated by Mef2.

Rather than potentially making an incorrect gene assignment, we did not assign the remaining 574 Mef2 bound fragments to genes, although they are equally significantly enriched with a stringent statistical cutoff. The vast majority (87.3%) of unassigned Mef2 bound fragments were in an intron or within 5 kb of one or more genes (distance score > 0.89); therefore, they likely contain regulatory modules. To enable other researchers to make their own gene assignments, we have created a searchable web site (http://furlonglab.embl.de/data/), displaying all Mef2 bound regions in their genomic context together with the results from our expression profiling experiments.

Mef2 Bound Regions Can Function as Muscle Enhancers In Vivo

Of the 1015 Mef2 bound regions, 62 were previously identified as active enhancers (Tables S4 and S5). The vast majority of these regulatory regions were not known to bind to Mef2, but they have been shown to bind to a number of other transcription factors in vitro, revealing interesting insights into combinatorial gene regulation with Mef2. For example, we identified Mef2 binding to an Antp bound enhancer region of the *apterous* and *teashirt* genes. Many of these regions were shown to function as muscle enhancers in vivo, providing additional evidence that our identified Mef2 bound regions are active enhancers. To test if additional Mef2 bound regions can function as enhancers in vivo, we selected five regions with representative levels of enrichment in the ChIP-on-chip experiments that were assigned to target genes with known expression. This allowed us to evaluate their ability to drive reporter gene expression in a pattern similar to that of the target gene. Using conservation in different *Drosophila* species as a guide, regions between 0.4 and 2.5 kb within the bound fragments were assayed.

All five Mef2 bound regions tested were able to drive GFP expression in *Mef2*-expressing cells. The enhancer region of *pnt* (Figure 2A″) initiates expression of GFP early in the mesoderm, at stages 9–10. This mirrors the expression of the *pnt* transcripts in wild-type embryos. Since the GFP protein has a long half-life in *Drosophila* embryos, it can subsequently be detected in differentiating myoblasts. The enhancer regions of *CG14687* (Figure 2B″) and *CG5080* (Figure 2C″) initiate GFP expression slightly later. The enriched region upstream of *CG14687* is sufficient to direct expression of GFP initially in the visceral muscle (stage 11) and later in the somatic muscle (stage 12), closely resembling the spatial and temporal expression pattern of the gene itself.

Myosin light chain 2 (*Mlc2*) is a target of Mef2 proteins in vertebrates (Chambers et al., 1994; Navankasattusas et al., 1992). Our data show that this regulation is conserved in flies. A Mef2 bound region 5′ of the *Mlc2* locus reproduces the gene's expression, driving GFP expression in differentiating somatic muscle cells from stage 13 onward (Figure 2D″). Finally, the endogenous expression of *CG9416* initiates in the longitudinal visceral muscle precursors at stage 10 (Figure 2E′) and in the somatic muscle at stage 13. A 372 bp enhancer region is sufficient to direct GFP expression in both tissues, reproducing the full expression pattern of *CG9416* (Figure 2E″).

In summary, all five Mef2 bound genomic regions are sufficient to direct reporter gene expression resembling the temporal and spatial patterns of the respective predicted target gene. These results indicate that our ChIP-on-chip approach was very successful in identifying new muscle enhancers in vivo, and that our assignment of target genes to Mef2 bound regions is accurate.

Mef2 Is Able to Direct Ectopic Expression of Novel Target Genes in the Ectoderm

Many of the identified direct target genes are misexpressed in *Mef2* mutant embryos, showing a requirement for Mef2 for their normal expression (see Supplemental Results). To determine if Mef2 is sufficient to regulate its target genes in nonmuscle cells, *Mef2* was ectopically expressed in the ectoderm by using the UAS/Gal4 system. As putative cofactors are likely to be absent, this assay is a very stringent test of a regulatory relationship. Remarkably, *Mef2* could induce ectopic expression of 5 of the 13 genes tested in nonmuscle cells. Overexpression of *Mef2* in *engrailed* stripes in the ectoderm is sufficient to cause ectopic expression of *Him*, *CG9416*, and *CG30080* (arrows, Figures 3A–3F). Overexpression of *Mef2* with *lmd*, a transcription factor known to regulate *Mef2* (Duan et al., 2001), is sufficient to drive ectopic expression of *CG5080* and *delilah* in the ectoderm (Figure 3; arrows in [G]–[J]). Ectopic expression of either transcription factor alone could not induce expression of these genes, suggesting that Mef2 and Lmd cooperatively regulate their expression and are sufficient to do so in nonmuscle cells. These results, in combination with the expression profiling data, confirm that *Mef2* is essential and sufficient for the expression of a large percentage of its target genes.

Mef2 Binds to Enhancers in Three Temporal Patterns during Development

While Mef2 is found in all muscle cells from gastrulation to the end of embryogenesis, its known target genes show temporally and spatially different expression

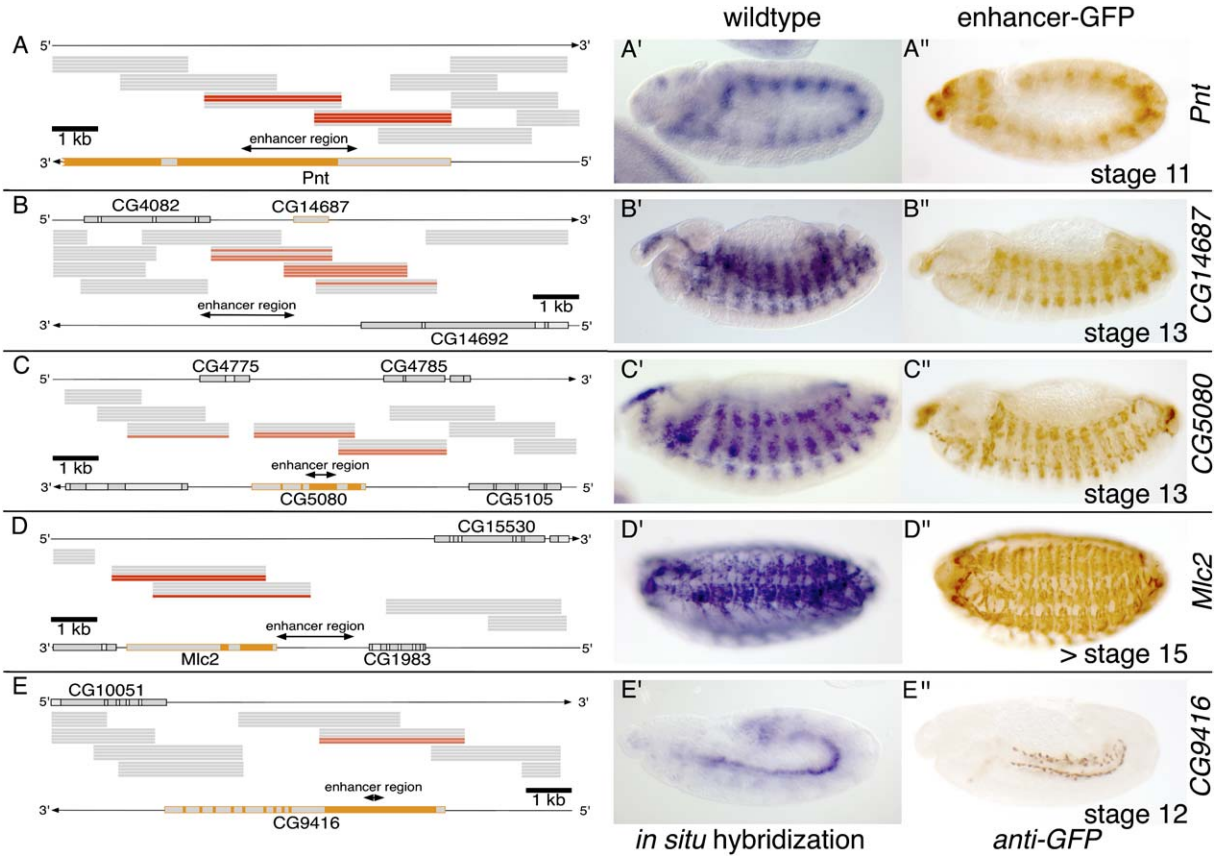

Figure 2. Mef2 Bound Fragments in ChIP-on-Chip Can Function as Muscle Enhancers In Vivo

(A–E) Schematic diagrams indicating the gene locus (orange), the genomic fragments on the tiling array (gray bars), the Mef2 bound fragments (red bars), and the enhancer region tested (arrowed line). The five gray or red bars in each stack represent the results for each developmental time point.

(A′–E′) In situ hybridization of transcripts in wild-type embryos.

(A″–E″) α-GFP immunohistochemistry of transgenic enhancer-GFP lines for the regions indicated in (A)–(E), respectively. Specific muscle staining reproducing the wild-type expression of the assigned target gene is observed in all enhancer lines tested, indicating that these Mef2 bound regions are sufficient to function as muscle enhancers in vivo. For all embryos, anterior is to the left and dorsal is to the top.

patterns. For example, *Act57B* and *β3-tub60D* RNA are not transcribed until ~stage 11, while *Mhc* and *Mlp84B* RNAs are not detectable until stages 13–14. Moreover, while *Mef2* is expressed in the entire myogenic lineage, some of its known targets are expressed in smaller subsets of cells.

Clearly, there must be additional ways to control Mef2's regulatory activity. To determine if regulation occurs at the level of DNA binding, we used the temporal information from the ChIP time course to investigate if there are distinct patterns of Mef2 enhancer occupancy. K-means clustering analysis was used to subdivide the 1015 enriched genomic regions according to their temporal profile of Mef2 binding. Three major groups of temporally bound enhancers were identified.

Binding to the first group of enhancers was initially detected at 4–6 hr of development, after which Mef2 remained bound through the three subsequent developmental time points assayed (Figure 4A, blue line, continuous cluster). This group, representing almost half of the enriched fragments, suggests that these enhancers remain occupied by Mef2 throughout development. This temporal binding pattern of Mef2 matches its broad expression during all stages of muscle development.

The second group, representing 21% of the enhancers, was bound by Mef2 at 4–6 hr of development, but it was not bound at later developmental time points (Figure 4A, green line, early cluster). As Mef2 continues to be expressed, and is capable of binding to other enhancers, the transient occupancy of the early enhancers demonstrates that Mef2's ability to bind to DNA is tightly regulated.

The third group, containing 32% of the enhancers, is only bound by Mef2 late in development, with maximal binding at the last time point assayed (Figure 4A, red line, late cluster). This group contains enhancers for many genes involved in late aspects of muscle differentiation, e.g., *Mhc*, *Mlc1*, *Mlc2*, *TmI*, *TmII*, *act57B*, *β3-tub60D*, *Mlp60A*, *Mlp84b*, *Mp20*, *mbl*, and *wupA*. Although Mef2 protein is present at high levels early in development, it does not occupy these enhancers until much later, demonstrating additional specificity in the regulation of Mef2 binding.

We investigated if the temporal binding of Mef2 to a target gene's enhancer coincides with the onset of that gene's expression. Remarkably, the first time point when Mef2 binds to an enhancer is significantly correlated with the onset of that gene's expression during

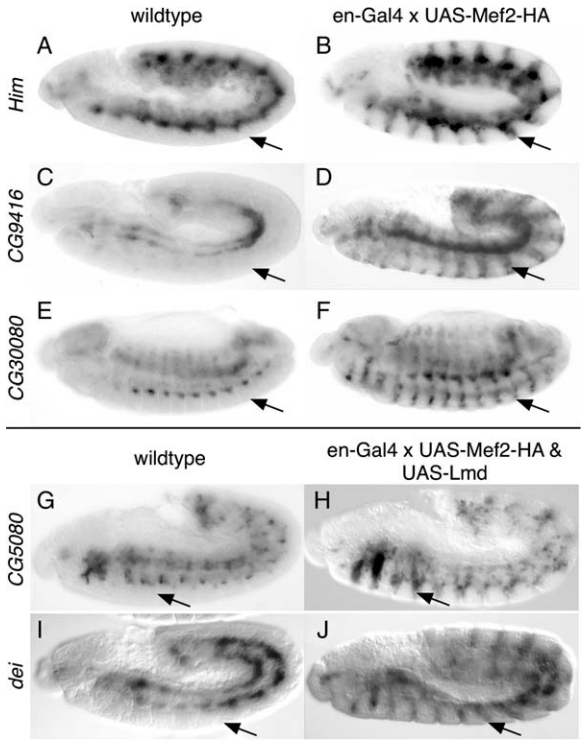

Figure 3. Ectopic *Mef2* Expression Can Induce Misexpression of Several Target Genes

(A–J) (A, C, E, G, and I) In situ hybridization of wild-type embryos with probes specific for (A) *Him*, (C) *CG9416*, (E) *CG30080*, (G) *CG5080*, and (I) *dei*, detecting specific expression in the visceral and somatic muscle. No specific staining was observed in the ectoderm (arrows). An engrailed-Gal4 driver line was used to ectopically express (B, D, and F) UAS-*Mef2*-HA or both (H and J) UAS-*Mef2*-HA and UAS-*lmd* in ectodermal stripes. The expression of all five genes can be induced in the ectoderm at stages 11–13 (arrows), either by (B, D, and F) ectopic Mef2 alone or (H and J) in combination with ectopic Lmd.

embryogenesis (Kendall's tau correlation, $p < 0.005$). While this trend holds for all time points assayed, the correlation is particularly strong for late bound enhancers (>10 hr), mirroring the coordinated expression of late muscle differentiation genes. Although additional levels of "post binding" regulation cannot be excluded, these results demonstrate that Mef2's DNA binding is tightly regulated and is a trigger for target gene expression.

To our knowledge, our results provide the first evidence that, while Mef2 is broadly expressed during muscle development, its ability to bind to DNA is temporally regulated depending on the context of the enhancer. This finding is intriguingly similar to what has been shown for MyoD in fibroblasts (Bergstrom et al., 2002) and Pha-4 in *C. elegans* (Gaudet et al., 2004). While both transcription factors have broad temporal expression, they regulate temporally restricted enhancers. This highlights a potentially general mechanism for encoding spatiotemporal specificity within the context of a regulatory region.

The Presence of Regulatory Motifs Correlates with Temporal Mef2 Binding

To explain the three temporal patterns of Mef2 binding, we searched for regulatory motifs within each group of enhancers. We first determined if the number of Mef2 sites was equally distributed between the three temporal groups of Mef2 bound enhancers. Interestingly, enhancers in the continuously bound and the late bound groups were significantly enriched in single and multiple Mef2 sites per fragment (Figure 4B). This significant enrichment of Mef2 sites is conserved in the ortologous sequences of the related species, *Drosophila pseudoobscura*. In contrast, the early cluster of transiently bound enhancers contains a similar number of Mef2 sites as the rest of the genome.

The bHLH transcription factor Twist is essential for all aspects of early mesoderm development in *Drosophila*. *Twist* has a transient expression pattern, with peak expression at stage 11 (~6–7 hr) mirroring the peak binding of Mef2 to the early enhancers (Figure 4A). In vitro studies have shown cooperative regulation between vertebrate Mef2 proteins and bHLH transcription factors via direct protein-protein intereactions (e.g., MyoD, Molkentin et al., 1995, and Hand, Morin et al., 2005). Given the temporal expression of Twist protein and the ability of vertebrate Mef2 proteins to bind to bHLH proteins, cooperative binding of Mef2 and Twist is an attractive model to explain the transient Mef2 binding to the early group of enhancers.

Two lines of evidence indicate that this hypothesis is correct. First, Twist sites are significantly enriched in the early bound enhancers (Figure 4C; $p < 10^{-4}$), and not in the continuous and late bound enhancers. This significant enrichment of Twist sites is conserved in the ortologous sequences of *D. pseudoobscura*. Second, Twist and Mef2 cobind to the early enhancers at the same stage of development. We have performed ChIP-on-chip studies of Twist at 4–6 hr of development (manuscript in preparation). Comparing the results of both studies showed in vivo binding of Twist to a large percentage of the early enhancers, demonstrating that Mef2 and Twist can cooccupy the early enhancers. Figure 4D shows five examples of Mef2 bound early enhancers cobound by Twist at 4–6 hr of development.

While bHLH proteins have a central role in muscle development in all species examined to date, the predominant roles of individual family members have diverged. In vertebrates, MyoD family members play a central role in activating muscle gene expression, while Twist represses myogenesis. In *Drosophila,* the only MyoD family member, *nau*, is not essential for general muscle development (Balagopalan et al., 2001). It has been speculated that Twist is the central bHLH regulator of *Drosophila* muscle development, as it is sufficient to activate the myogenic program upon ectopic expression in the ectoderm (Baylies and Bate, 1996). Our results provide further evidence for the evolutionary similar roles of Twist in flies and vertebrate MRFs; *Drosophila* Twist and Mef2 proteins may cooperatively regulate muscle gene expression in a similar manner to MyoD and Mef2 proteins in vertebrates.

Examination of Mef2 Target Genes Reveals New Insights into the Role of Mef2 in Muscle Development

Mef2 Coordinates Multiple Processes of Terminal Muscle Differentiation

Embryos with loss-of-function mutations in *Drosophila Mef2* show a block of myoblast fusion and lack

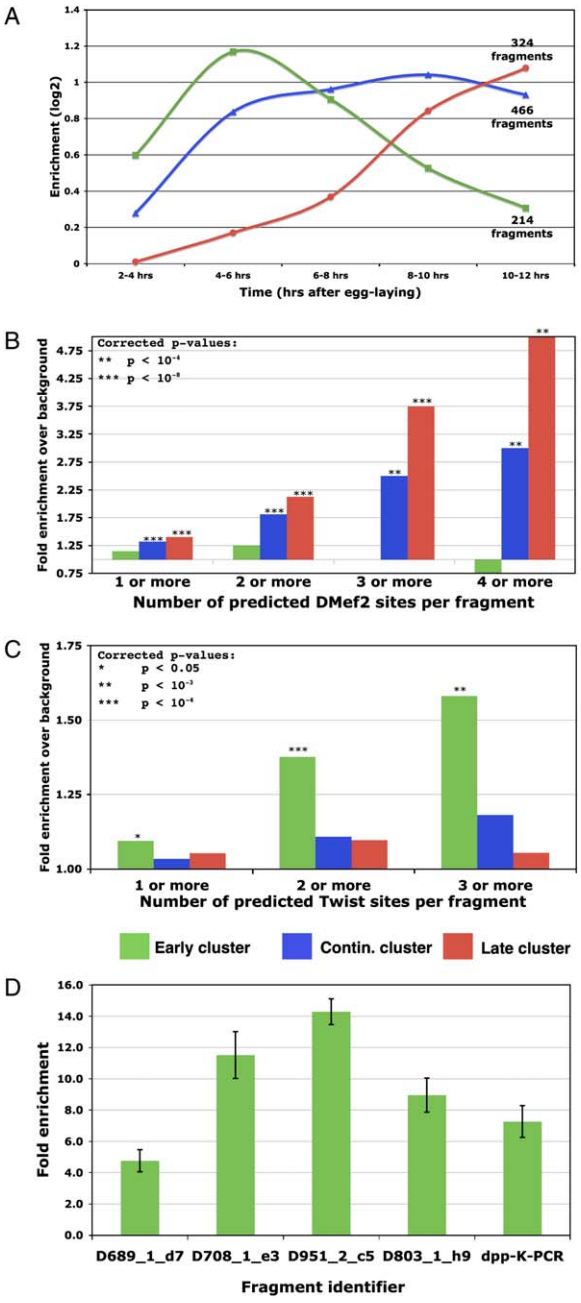

Figure 4. Mef2 Binds to Three Temporal Classes of Regulatory Regions

(A) K-means nearest neighbor clustering identified three major temporal patterns of Mef2 binding to 1004 enriched regions. Each line in the graph represents the median enrichment for all fragments within that group at a given time point. The early bound cluster is in green, the continuously bound cluster is in blue, and the late bound cluster is in red.

(B) Mef2 binding sites are overrepresented in the enriched regions: using a position weight matrix (PWM) for Mef2, a significant enrichment of one or more predicted binding sites was found in the continuously bound (blue) and late bound (red) fragments (***$p < 10^{-8}$). The y axis shows fold enrichment of the number of Mef2 sites in the enriched fragments, compared to the rest of the fragments on the array (background).

(C) Twist binding sites are enriched in the early bound cluster: using a PWM to predict Twist binding sites, a significant enrichment of one or more sites per fragment was found only in the early bound cluster (green) (*$p < 0.05$, **$p < 10^{-3}$, ***$p < 10^{-4}$).

expression of a number of contractile muscle proteins (Bour et al., 1995; Lilly et al., 1995). We have identified a number of Mef2 target genes involved in both processes; e.g., *blow* and *lmd*, two genes essential for myoblast fusion, as well as numerous cytoskeletal proteins (Table S7).

In addition to these severe phenotypes, defects in neuromuscular junction (NMJ) formation and muscle attachments have been observed in *Mef2* mutant embryos (Prokop et al., 1996; Ranganayakulu et al., 1995), the molecular basis of which is not understood. We find a significant enrichment of Mef2 target genes involved in both processes (Figure 5A, $p < 0.01$; Table S8), providing a molecular understanding of the observed phenotypes.

Our results also indicate that Mef2 is required for muscle function in differentiated myofibers. We identified a number of Mef2 target genes involved in muscle energy production or storage: *Pfrx*, *GluRIIA*, *GlyP*, *Gpdh*, *Glycogenin*, *Pgi*, and *Pgk*. This role may be further strengthened by the direct regulation of Ptx1, a transcription factor thought to regulate muscle physiology (Vorbruggen et al., 1997).

An Early Role for Mef2 in the Regulation of Muscle Identity Genes

Drosophila body wall muscles are formed from progenitor cells that are selected through the action of Ras signaling and Notch-Delta lateral inhibition (reviewed by Furlong, 2004). Our data show in vivo binding of Mef2 to enhancer regions of a striking number of genes that are essential for this process (Figure 6; red border), in addition to expression changes for some genes in *Mef2* mutant embryos (Figure 6; blue or yellow). This includes components of the Notch-Delta pathway (*Delta*, *mam*, *bib*, *E(spl)* complex, and *Neur*), which is essential for specification of the somatic muscle and heart. As our experiments were analyzed with tiling arrays covering 50% of the genome, Mef2 likely regulates even more genes involved in these signaling pathways, which we could not identify. This is in agreement with Junion et al. (2005), who identified *sfl*, *spen*, and *argos* as Mef2 targets.

Once specified, founder cells express a characteristic set of transcription factors called identity genes. We identified in vivo binding of Mef2 to enhancer regions of eight of the ten known muscle identity genes (Figure 6, green box; Figure S4). Importantly, the temporal binding of Mef2 correlates with the initiation of identity gene expression in founder cells (stages 9–11; time points 4–6 and 6–8 hr). Mef2 also binds to enhancer regions of a number of genes previously reported to be enriched in founder cells, suggesting that Mef2 acts in concert with the identity genes to regulate the transcriptional program within these myoblasts (*Ubx*, *htl*, *CG14207*, *CG9520*, *CG17492*, *CG8417*, Artero et al., 2003, and krT95D in the VO5 muscle, Hartmann and Jackle, 1997).

Genetic studies looking at the interplay between motor neuron and muscle development observed a consistent reduction in the number of somatic muscles in *Mef2*

(D) Twist cobinds to Mef2-early bound enhancers: chromatin immunoprecipitation was performed with an anti-Twist antibody at 4–6 hr of development and was assayed by real-time PCR. Examples of five sequences from the early Mef2-enriched cluster are shown to be cobound by Twist (n = 2). Cooperative binding of Twist and Mef2 is likely regulating the transient binding of Mef2 to these enhancers.

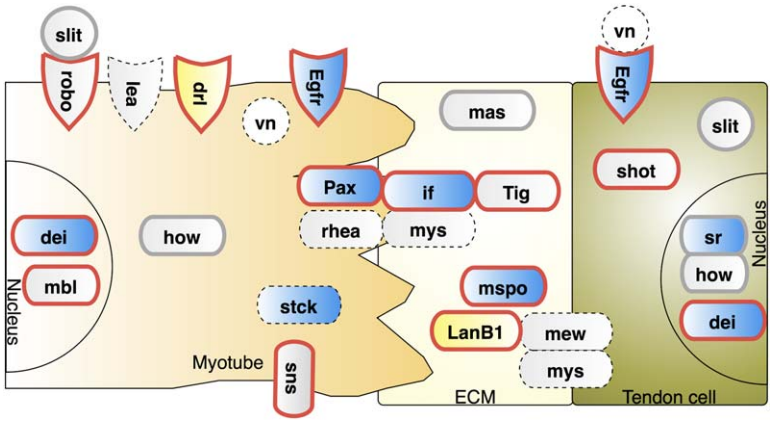

Figure 5. Mef2 Directly Regulates Many Genes Involved in Muscle Attachment

Depicted are a myotube on the left, a tendon cell on the right, and the extracellular matrix (ECM) in between. Gene products are represented in the expressing cell or in the ECM. The color of the border of each gene represents the results from the ChIP-on-chip experiment: genes with a thick, red border are bound by Mef2, genes with a thin, black, dashed border are not covered by the tiling array, and a thick, gray border indicates coverage on the array, but the loci are not bound by Mef2. Blue and yellow represent the results from expression profiling of *Mef2* mutant embryos, indicating reduced or increased expression, respectively. No gene expression information for *vn* is available.

mutants (Landgraf et al., 1999; Prokop et al., 1996). At that time, it was not clear if the loss of muscles was a secondary defect due to a general failure of the muscle to differentiate, and the molecular mechanism was not explored. Our results indicate that Mef2 directly regulates founder cell identity gene expression. We suggest that Mef2 provides an extra layer of regulation to buffer this key step in muscle development from stochastic fluctuations in the levels of key regulators. When this regulation is absent in *Mef2* mutant embryos, founder cells are not specified or maintained in a robust manner, leading to the observed low penetrance loss-of-muscles phenotype.

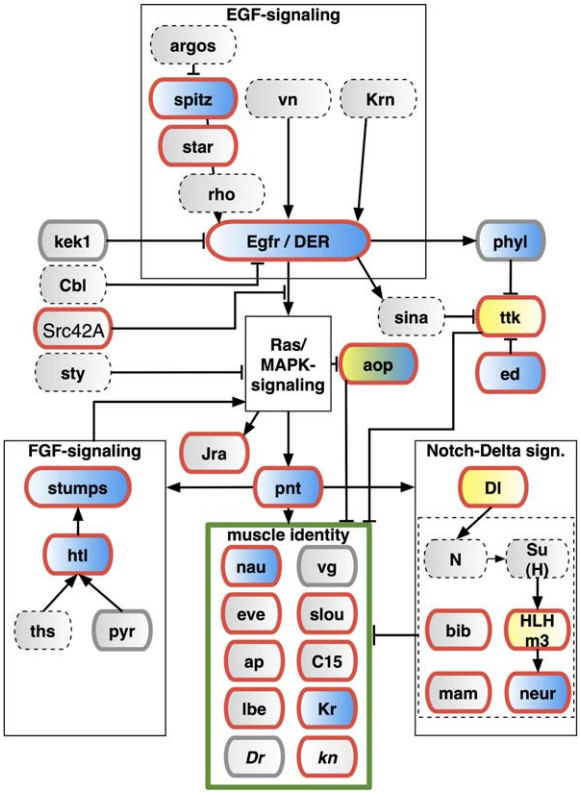

Figure 6. A New Role for Mef2: Regulation of Muscle Identity

A schematic overview of genes involved in somatic muscle specification: The EGF-, FGF-, and Notch-Delta signaling pathways converge to specify progenitor cells, which give rise to somatic muscle founder cells. Founder cells express specific transcription factors or identity genes. The colors used represent the results from ChIP-on-chip (border) and expression profiling experiments (fill). The loci of genes with a thick, red border were bound by Mef2, the loci of genes with a thick, gray border were not bound by Mef2, and the loci of genes with a thin, black, dashed border are not covered by the tiling array. Blue and yellow represent the results from expression profiling of *Mef2* mutant embryos, indicating reduced or increased expression, respectively. Individual components of the Ras/MAPK pathway (central square box) have been omitted for clarity.

Mef2 Is a Key Regulator of the Transcriptional Circuitry Required for Muscle Development

Traditionally, Mef2 is placed toward the bottom of the myogenic transcriptional hierarchy, due to its well-characterized role as a regulator of muscle effector proteins. Because of this, we were very surprised to find that Mef2 regulates a large number of transcription factors, many of which are involved in early aspects of myogenesis (Supplemental Results; Table S9). For example, Mef2 regulates transcription factors essential for visceral muscle development (*bap*, *slp1*, *HLH54F*) and cardioblast specification (*nmr2*, *Zfh1*, *ush*). These results implicate a role for Mef2 in the subdivision of the dorsal mesoderm and place Mef2 at the center of the transcriptional program required for *Drosophila* muscle development.

Vertebrate Mef2 proteins regulate the expression of the transcription factors MyoD and cJun (Han and Prywes, 1995; Wong et al., 1994). We find direct regulation of the orthologs of these genes (*nau*, *jra*) by *Drosophila* Mef2, indicating that Mef2 is part of an evolutionary conserved genetic program. This suggests that many of the additional transcriptional connections that we have identified in *Drosophila* may also be conserved.

A New View of Mef2 as a Global Regulator of Muscle Gene Expression in Drosophila

One of the most surprising findings of this study was the large number of enhancer regions that are bound by Mef2. Previous studies searching for Mef2 targets focused on a limited part of the genome. To our knowledge, the present study is the most comprehensive and unbiased report to date and thereby provides a unique opportunity to get a more accurate view of the total number of Mef2-regulated enhancers and genes. Using a tiling array covering ~50% of the genome, we have identified at least 670 unique Mef2 bound genomic regions. A total of 600 of these enhancer regions are within 5 kb of a gene

locus and are therefore likely to represent active enhancers of a gene. Extrapolating to the whole genome, this indicates that Mef2 regulates as many as 1000 genes during the course of muscle development.

The current view of Mef2 in the literature is of a transcription factor required late in development for muscle differentiation. However, given the diversity of Mef2 target genes, its ability to regulate genes during all stages of muscle development, and the huge number of enhancer regions bound by Mef2, our view of the function of the transcription factor needs to be adjusted. Mef2 is likely to bind to enhancer regions of most, if not all, muscle genes, not just structural muscle proteins, and may thereby act as a "general" muscle transcription factor. The presence of feed-forward loops regulated by Mef2 (see Supplemental Results) as well as of low-penetrance phenotypes in *Mef2* mutants suggests that one of the functions of this transcription factor is to provide robustness within the myogenic program.

Concluding Remarks

We present a systematic analysis of Mef2 activity during a time course of *Drosophila* embryogenesis by using two complementary genomic approaches. This unique combination provides insights into the function of this transcription factor at a number of levels: the identification of a large number of Mef2 bound enhancers provides a first step to decipher the regulatory network governing myogenesis. The dynamic aspect of Mef2 DNA binding reveals mechanistic insight into the regulation of Mef2's activity. The identification of Mef2's direct target genes allows a molecular understanding of the complex phenotypes observed in *Mef2* mutants. Finally, the integration of these data provides a new view of this transcription factor as a broad modulator of muscle gene expression.

This combination of positional and temporal information, derived from genomic approaches, can be applied to any transcription factor. Such studies on other regulators will allow the identification of overlapping and distinct enhancer regions, which are used dynamically during discrete stages of development. This combinatorial network of transcription factor activities is essential to elucidate the regulatory code required for complex developmental programs.

Experimental Procedures

Chromatin Immunoprecipitation and DNA Amplification

A detailed description of the chromatin immunoprecipitation method can be found in the Supplemental Data. In brief, for each time point studied, four independent staged wild-type embryo populations were collected. The chromatin samples were prepared from tightly staged 2 hr embryo collections (Table S1). Antisera were raised against amino acids 1–294 of dMef2 isoform IV (Gunthorpe et al., 1999), which was purified from *E. coli* with poly-His tag affinity purification. Sera from two different rabbits were used to exclude serum-dependent biases. Two ChIP replicates were performed with α-Mef2 antibody-A, and two were performed with α-Mef2 antibody-B (Figure 1B). Mock immunoprecipitations were performed by using preimmunesera on each chromatin sample, leading to a total of eight reactions (4 mock, 4 α-dMef2) per time point. Dye swaps were included to account for possible dye biases. The DNA labeling and hybridizations were performed as described in the Supplemental Data.

Expression Profiling of *Mef2* Loss-of-Function Mutants

The assayed $Mef2^{22.21}$ (Bour et al., 1995) line was generated by outcrossing the original line twice to wild-type flies (Canton S). One hr embryo collections of *Mef2* mutants and Canton S were collected and aged together, as described in Furlong et al., (2001a; 2001b). After selecting the homozygous mutants with an automated embryo sorter, any unfertilized embryos were subsequently removed by hand. The staging of all collections was verified by formaldehyde fixation of a small sample to ensure that wild-type and mutant embryos were tightly stage matched (Table S6). For each developmental time point assayed, four independent embryo collections, sorts, and hybridizations were performed. A detailed protocol of the RNA amplification, labeling, and hybridization is available in the Supplemental Data.

Analysis of Array Data

The raw data from both tiling and cDNA arrays were normalized by using print-tip and LOWESS normalization. To identify ChIP-enriched fragments, the experimental samples were hybridized against genomic DNA. The four mock/genomic DNA and four ChIP/genomic DNA ratios were compared in a two-class SAM analysis (Tusher et al., 2001). The false-discovery rate was estimated by calculating q values. Only fragments with $q < 0.01$ and a fold enrichment > 1.6 ($\log_2 > 0.7$) were considered to be significantly enriched.

To identify differentially expressed genes in *Mef2* mutant embryos, the experimental *Mef2* mutant samples were hybridized directly against the stage-matched wild-type samples. A one-class SAM analysis was performed on four independent biological repeats for each time point. Genes with a $q < 0.05$ and a fold enrichment of $\log_2 > 0.7$ or < -0.7 at two consecutive time points were considered to be differentially regulated.

The assignment of ChIP-enriched fragments to target genes is described in detail in the Supplemental Data. Mef2 binding and mutant expression data can be visualized by using a searchable web site (http://furlonglab.embl.de/data/).

Generation of Transgenic Reporter Strains

Fragments within the following coordinates were cloned into the pH-stinger vector (Barolo et al., 2000) for germline transformation: chr3R:26000269–26001748 (*Mlc2*), chr3R:6616700–6618790 (*CG14687*), chr2L:1162075–1163021 (*CG5080*), chr2R:14886267–14886639 (*CG9416*), chr3R:19167526–19169991 (*pnt*) (coordinates based on genome release 4.2). For all constructs, except *CG5080*'s regulatory region, at least two independent transgenic lines were obtained and assayed.

To ectopically express *Mef2* under the control of the UAS/Gal4 system, *Mef2* cDNA isoform IV (Gunthorpe et al., 1999) was fused to 3HA-epitope tags at the 3′ end and cloned into the pUAST vector (Brand and Perrimon, 1993). The presence of the epitope tag did not lead to any discernable difference from the described UAS-D*Mef2* lines. The UAS-*lmd* line has been described previously as UAS-*gfl* (Furlong et al., 2001a).

Histological Techniques

In situ hybridizations were done as described previously (Furlong et al., 2001a). The following ESTs were used to generate DIG-labeled probes: GH22991 (*delilah*), LD34147 (*CG5080*), RE70039 (*Him*), RE28322 (*CG9416*), and RE42467 (*CG30080*). The probe for *Mlc2* was generated from the largest exon (chr3R:25,998,722-25,999,116). GFP expression in transgenic animals was detected by immunohistochemistry with rabbit α-GFP antibody (Torrey Pines Biolabs) at a concentration of 1:500. Biotinylated secondary antibodies were used in combination with the Vector Elite ABC kit (Vector Laboratories).

Estimating Overrepresentation of Binding Sites in Pulled-Down Fragments

A detailed description is available in Supplemental Data. In brief, known binding sites for Mef2 and Twist were used to generate position-specific scoring matrices by using MEME. The MAST software was used to predict binding sites in the Mef2 bound fragments as well as in all fragments covered on the array (background) (Bailey and Gribskov, 1998). The significant enrichment of predicted sites in the enriched fragments was estimated by using Fisher's exact

test, and the p values were adjusted for multiple testing by using the Benjamini and Hocherg method.

Supplemental Data

Supplemental Data include all Mef2 bound regions, gene assignments, expression profiling data, graphical representations of known Mef2 bound enhancers, protocols, and methods and are available at http://www.developmentalcell.com/cgi/content/full/10/6/797/DC1/.

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References

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., Amanatides, P.G., Scherer, S.E., Li, P.W., Hoskins, R.A., Galle, R.F., et al. (2000). The genome sequence of *Drosophila melanogaster*. Science *287*, 2185–2195.

Artero, R., Prokop, A., Paricio, N., Begemann, G., Pueyo, I., Mlodzik, M., Perez-Alonso, M., and Baylies, M.K. (1998). The muscleblind gene participates in the organization of Z-bands and epidermal attachments of *Drosophila* muscles and is regulated by Dmef2. Dev. Biol. *195*, 131–143.

Artero, R., Furlong, E.E., Beckett, K., Scott, M.P., and Baylies, M. (2003). Notch and Ras signaling pathway effector genes expressed in fusion competent and founder cells during *Drosophila* myogenesis. Development *130*, 6257–6272.

Bagni, C., Bray, S., Gogos, J.A., Kafatos, F.C., and Hsu, T. (2002). The *Drosophila* zinc finger transcription factor CF2 is a myogenic marker downstream of MEF2 during muscle development. Mech. Dev. *117*, 265–268.

Bailey, T.L., and Gribskov, M. (1998). Combining evidence using p-values: application to sequence homology searches. Bioinformatics *14*, 48–54.

Balagopalan, L., Keller, C.A., and Abmayr, S.M. (2001). Loss-of-function mutations reveal that the *Drosophila* nautilus gene is not essential for embryonic myogenesis or viability. Dev. Biol. *231*, 374–382.

Barolo, S., Carver, L.A., and Posakony, J.W. (2000). GFP and β-galactosidase transformation vectors for promoter/enhancer analysis in *Drosophila*. Biotechniques *29*, 726, 728, 730, 732.

Baylies, M.K., and Bate, M. (1996). twist: a myogenic switch in *Drosophila*. Science *272*, 1481–1484.

Bergman, C.M., Carlson, J.W., and Celniker, S.E. (2005). *Drosophila* DNase I footprint database: a systematic genome annotation of transcription factor binding sites in the fruitfly, *Drosophila melanogaster*. Bioinformatics *21*, 1747–1749.

Bergstrom, D.A., Penn, B.H., Strand, A., Perry, R.L., Rudnicki, M.A., and Tapscott, S.J. (2002). Promoter-specific regulation of MyoD binding and signal transduction cooperate to pattern gene expression. Mol. Cell *9*, 587–600.

Black, B.L., and Olson, E.N. (1998). Transcriptional control of muscle development by myocyte enhancer factor-2 (MEF2) proteins. Annu. Rev. Cell Dev. Biol. *14*, 167–196.

Blais, A., Tsikitis, M., Acosta-Alvear, D., Sharan, R., Kluger, Y., and Dynlacht, B.D. (2005). An initial blueprint for myogenic differentiation. Genes Dev. *19*, 553–569.

Bour, B.A., O'Brien, M.A., Lockwood, W.L., Goldstein, E.S., Bodmer, R., Taghert, P.H., Abmayr, S.M., and Nguyen, H.T. (1995). *Drosophila* MEF2, a transcription factor that is essential for myogenesis. Genes Dev. *9*, 730–741.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401–415.

Chambers, A.E., Logan, M., Kotecha, S., Towers, N., Sparrow, D., and Mohun, T.J. (1994). The RSRF/MEF2 protein SL1 regulates cardiac muscle-specific transcription of a myosin light-chain gene in *Xenopus* embryos. Genes Dev. *8*, 1324–1334.

Cripps, R.M., Lovato, T.L., and Olson, E.N. (2004). Positive autoregulation of the Myocyte enhancer factor-2 myogenic control gene during somatic muscle development in *Drosophila*. Dev. Biol. *267*, 536–547.

Damm, C., Wolk, A., Buttgereit, D., Loher, K., Wagner, E., Lilly, B., Olson, E.N., Hasenpusch-Theil, K., and Renkawitz-Pohl, R. (1998). Independent regulatory elements in the upstream region of the *Drosophila* beta 3 tubulin gene (beta Tub60D) guide expression in the dorsal vessel and the somatic muscles. Dev. Biol. *199*, 138–149.

Duan, H., Skeath, J.B., and Nguyen, H.T. (2001). *Drosophila* Lame duck, a novel member of the Gli superfamily, acts as a key regulator of myogenesis by controlling fusion-competent myoblast development. Development *128*, 4489–4500.

Furlong, E.E. (2004). Integrating transcriptional and signalling networks during muscle development. Curr. Opin. Genet. Dev. *14*, 343–350.

Furlong, E.E., Andersen, E.C., Null, B., White, K.P., and Scott, M.P. (2001a). Patterns of gene expression during *Drosophila* mesoderm development. Science *293*, 1629–1633.

Furlong, E.E., Profitt, D., and Scott, M.P. (2001b). Automated sorting of live transgenic embryos. Nat. Biotechnol. *19*, 153–156.

Gallo, S.M., Li, L., Hu, Z., and Halfon, M.S. (2006). REDfly: a regulatory element database for *Drosophila*. Bioinformatics *22*, 381–383.

Gaudet, J., Muttumu, S., Horner, M., and Mango, S.E. (2004). Whole-genome analysis of temporal gene expression during foregut development. PLoS Biol. *2*, e352.

Gunthorpe, D., Beatty, K.E., and Taylor, M.V. (1999). Different levels, but not different isoforms, of the *Drosophila* transcription factor DMEF2 affect distinct aspects of muscle differentiation. Dev. Biol. *215*, 130–145.

Han, T.H., and Prywes, R. (1995). Regulatory role of MEF2D in serum induction of the c-jun promoter. Mol. Cell. Biol. *15*, 2907–2915.

Hartmann, C., and Jackle, H. (1997). Putative Kruppel target gene of novel sequence expressed in *Drosophila* VO5 muscle precursors. Dev. Genes Evol. *207*, 186–207.

Junion, G., Jagla, T., Duplant, S., Tapin, R., Da Ponte, J.P., and Jagla, K. (2005). Mapping Dmef2-binding regulatory modules by using a ChIP-enriched in silico targets approach. Proc. Natl. Acad. Sci. USA *102*, 18479–18484.

Kelly, K.K., Meadows, S.M., and Cripps, R.M. (2002). *Drosophila* MEF2 is a direct regulator of Actin57B transcription in cardiac, skeletal, and visceral muscle lineages. Mech. Dev. *110*, 39–50.

Landgraf, M., Baylies, M., and Bate, M. (1999). Muscle founder cells regulate defasciculation and targeting of motor axons in the *Drosophila* embryo. Curr. Biol. *9*, 589–592.

Lilly, B., Zhao, B., Ranganayakulu, G., Paterson, B.M., Schulz, R.A., and Olson, E.N. (1995). Requirement of MADS domain transcription factor D-MEF2 for muscle formation in *Drosophila*. Science *267*, 688–693.

Lin, M.H., Bour, B.A., Abmayr, S.M., and Storti, R.V. (1997a). Ectopic expression of MEF2 in the epidermis induces epidermal expression of muscle genes and abnormal muscle development in *Drosophila*. Dev. Biol. *182*, 240–255.

Lin, Q., Schwarz, J., Bucana, C., and Olson, E.N. (1997b). Control of mouse cardiac morphogenesis and myogenesis by transcription factor MEF2C. Science *276*, 1404–1407.

Lin, S.C., and Storti, R.V. (1997). Developmental regulation of the *Drosophil*a Tropomyosin I (Tml) gene is controlled by a muscle activator enhancer region that contains multiple cis-elements and binding sites for multiple proteins. Dev. Genet. *20*, 297–306.

Molkentin, J.D., Black, B.L., Martin, J.F., and Olson, E.N. (1995). Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. Cell *83*, 1125–1136.

Morin, S., Pozzulo, G., Robitaille, L., Cross, J., and Nemer, M. (2005). MEF2-dependent recruitment of the HAND1 transcription factor results in synergistic activation of target promoters. J. Biol. Chem. *280*, 32272–32278.

Navankasattusas, S., Zhu, H., Garcia, A.V., Evans, S.M., and Chien, K.R. (1992). A ubiquitous factor (HF-1a) and a distinct muscle factor (HF-1b/MEF-2) form an E-box-independent pathway for cardiac muscle gene expression. Mol. Cell. Biol. *12*, 1469–1479.

Naya, F.J., Black, B.L., Wu, H., Bassel-Duby, R., Richardson, J.A., Hill, J.A., and Olson, E.N. (2002). Mitochondrial deficiency and cardiac sudden death in mice lacking the MEF2A transcription factor. Nat. Med. *8*, 1303–1309.

Nguyen, H.T., Bodmer, R., Abmayr, S.M., McDermott, J.C., and Spoerel, N.A. (1994). D-mef2: a *Drosophila* mesoderm-specific MADS box-containing gene with a biphasic expression profile during embryogenesis. Proc. Natl. Acad. Sci. USA *91*, 7520–7524.

Paris, J., Virtanen, C., Lu, Z., and Takahashi, M. (2004). Identification of MEF2-regulated genes during muscle differentiation. Physiol. Genomics *20*, 143–151.

Penn, B.H., Bergstrom, D.A., Dilworth, F.J., Bengal, E., and Tapscott, S.J. (2004). A MyoD-generated feed-forward circuit temporally patterns gene expression during skeletal muscle differentiation. Genes Dev. *18*, 2348–2353.

Prokop, A., Landgraf, M., Rushton, E., Broadie, K., and Bate, M. (1996). Presynaptic development at the *Drosophila* neuromuscular junction: assembly and localization of presynaptic active zones. Neuron *17*, 617–626.

Ranganayakulu, G., Zhao, B., Dokidis, A., Molkentin, J.D., Olson, E.N., and Schulz, R.A. (1995). A series of mutations in the D-MEF2 transcription factor reveal multiple functions in larval and adult myogenesis in *Drosophila*. Dev. Biol. *171*, 169–181.

Sokol, N.S., and Ambros, V. (2005). Mesodermally expressed *Drosophila* microRNA-1 is regulated by Twist and is required in muscles during larval growth. Genes Dev. *19*, 2343–2354.

Stronach, B.E., Renfranz, P.J., Lilly, B., and Beckerle, M.C. (1999). Muscle LIM proteins are associated with muscle sarcomeres and require dMEF2 for their expression during *Drosophila* myogenesis. Mol. Biol. Cell *10*, 2329–2342.

Taylor, M.V. (2000). A novel *Drosophila*, mef2-regulated muscle gene isolated in a subtractive hybridization-based molecular screen using small amounts of zygotic mutant RNA. Dev. Biol. *220*, 37–52.

Tusher, V.G., Tibshirani, R., and Chu, G. (2001). Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. USA *98*, 5116–5121.

Vorbruggen, G., Constien, R., Zilian, O., Wimmer, E.A., Dowe, G., Taubert, H., Noll, M., and Jackle, H. (1997). Embryonic expression and characterization of a Ptx1 homolog in *Drosophila*. Mech. Dev. *68*, 139–147.

Wang, L., Fan, C., Topol, S.E., Topol, E.J., and Wang, Q. (2003). Mutation of MEF2A in an inherited disorder with features of coronary artery disease. Science *302*, 1578–1581.

Wong, M.W., Pisegna, M., Lu, M.F., Leibham, D., and Perry, M. (1994). Activation of *Xenopus* MyoD transcription by members of the MEF2 protein family. Dev. Biol. *166*, 683–695.

Accession Numbers

All microarray data are available from ArrayExpress (accession codes E-TABM-56 and E-TABM-57) and from http://furlonglab.embl.de/data/.