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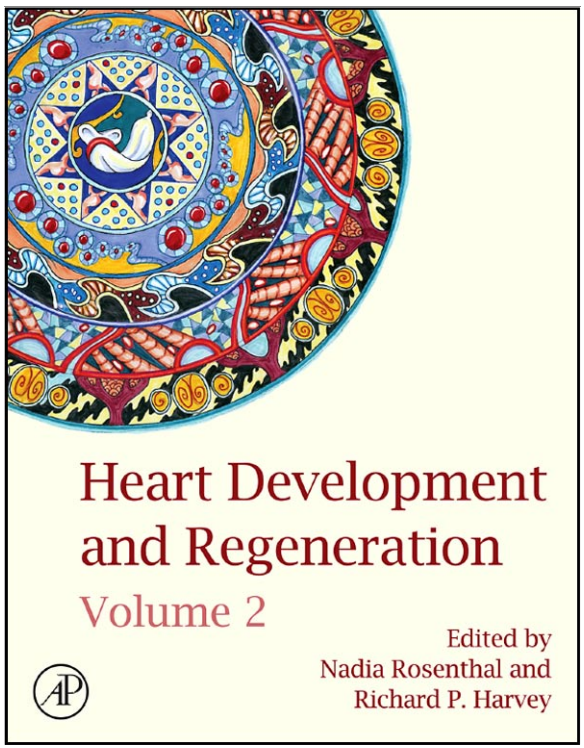

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Chapter 9.5

Myocyte Enhancer Factor 2 Transcription Factors in Heart Development and Disease

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I. INTRODUCTION

Members of the myocyte enhancer factor 2 (MEF2) family of transcription factors are important regulators of gene expression in numerous tissues, including the heart, where MEF2 plays important roles in development and in postnatal adaptation to a wide array of physiological and pathological signals. MEF2 functions as a transcriptional switch, by potently activating or repressing transcription through interaction with a variety of co-factors which serve as positive and negative regulators of transcription. The interaction of MEF2 with its co-factors is controlled by a multitude of signaling pathways that result in post-translational modification of MEF2, and in the subsequent MEF2-dependent repression or activation of target gene transcription. This allows MEF2 to link the extracellular environment to distinct and highly-regulated transcriptional outputs through intracellular signaling cascades and co-factor interactions. In the heart, MEF2 is essential for development and plays fundamental roles in myocyte differentiation and gene activation. MEF2 is also crucial in the postnatal heart for integrating the transcriptional response to numerous environmental cues, and regulating normal physiological and pathological growth and adaptation of the heart.

In this chapter, we review what is known about the general regulation and function of MEF2 transcription factors, with a focus on their role in the heart. We discuss the many co-factors of MEF2 with a particular attention to the interaction of MEF2 with class II histone deacetylases (HDACs) (see Chapter 10.2). MEF2-HDAC interactions are highly-regulated by signaling cascades that control MEF2's function as a transcriptional switch. We highlight the many signaling pathways and kinases that regulate MEF2-HDAC interactions, MEF2 post-translational modification, and how these pathways influence MEF2 activity. We also discuss the genetic function of *Mef2* genes in flies, fish and mice. These studies demonstrate the essential function for MEF2 proteins in heart development, as well as the development of numerous other lineages, and reflect the general conservation of MEF2 function throughout much of metazoan evolution. Many genes have been identified as direct transcriptional targets of MEF2 through direct MEF2 binding to their promoter and enhancer elements, and we summarize the known direct transcriptional targets of MEF2 in the heart.

The *Mef2* genes themselves are regulated at the transcriptional level by the activity of multiple, independent modular enhancers. These discrete enhancer modules control *Mef2* expression in a restricted subset of the gene's complete expression pattern. We review the transcriptional regulation of the single *Mef2* gene in *Drosophila* and the mouse *Mef2c* gene, which has been the best-characterized vertebrate *Mef2* gene in terms of transcriptional regulation. These studies have uncovered many new roles for *Mef2* genes in the heart and other tissues by identifying unexpected expression patterns and regulatory interactions upstream of MEF2. Finally, in this chapter, we highlight several important areas for future investigation regarding the role of MEF2 transcription factors, how they are regulated and function in the developing and postnatal heart, and their possible involvement in human cardiovascular disease.

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II. THE MEF2 FAMILY OF TRANSCRIPTION FACTORS

II.A. Discovery of MEF2 Transcription Factors

In the late-1980s and early-1990s, numerous transcription factors involved in skeletal muscle development were identified. These studies were highlighted by the seminal observations of Davis et al. who showed that the myogenic basic helix-loop-helix (bHLH) transcription factor MyoD alone could initiate skeletal myogenesis in a broad range of cell types in culture (Davis et al., 1987). MEF2 proteins were also identified during this time as essential regulators of skeletal muscle transcription, and as partners for MyoD and other myogenic bHLH proteins. In addition, it soon became apparent that MEF2 was a critical regulator of muscle development in all muscle lineages, including the heart (Black and Olson, 1998).

MEF2 transcription factors were discovered independently using two different approaches. On the one hand, several groups had determined that muscle cells contained different DNA-binding activities that could interact with muscle structural gene promoter sequences *in vitro* and *in vivo*. MEF2-binding activity interacted strongly and specifically with an AT-rich sequence that was found in the promoters of numerous muscle-specific genes, and the integrity of these sites was required for full muscle-specific gene activation (Gossett et al., 1989; Horlick and Benfield, 1989; Mueller and Wold, 1989). Meanwhile, Treisman and colleagues were studying the function of the serum response factor (SRF) protein. Serum response factor had been shown to be a potent activator of genes important to proliferation, as well as to differentiation of vascular muscle lineages, interacting with DNA via a conserved binding domain (Treisman, 1990). By screening for cDNAs similar to that of serum response factor, Pollock and Treisman identified factors named Related to SRF4 (RSRF4, now called MEF2D) and RSRFR2 (MEF2B) (Pollock and Treisman, 1991). These investigators went on to demonstrate that RSRF proteins probably corresponded to the muscle-specific binding activities previously defined as MEF2. Using a similar approach, Chambers et al. (1992) identified *Xenopus* SL-1 (MEF2D) and SL-2 (MEF2A).

A more direct connection between MEF2 binding activity and the RSRF proteins was achieved by Yu et al. (1992), who used a concatamerized MEF2 binding site from the muscle creatine kinase (*MCK*) gene to screen a cDNA expression library for factors that bound to the MEF2 sequence. This resulted in the identification of human MEF2A. The final MEF2 family member, MEF2C, was cloned based on its similarity to existing MEF2 factors, and was the first to show significant enrichment in muscle tissues in the developing embryo (Martin et al., 1993; Edmondson et al., 1994). It is now well-established that most vertebrate genomes contain at least four MEF2-encoding genes, whereas simpler animals such as *Drosophila melanogaster*, *Caenorhabditis elegans* and *Ciona intestinalis* contain only a single *Mef2* gene each (Olson et al., 1995; Davidson, 2007).

II.B. The MEF2 Family in the Context of the MADS Domain Superfamily

MEF2 proteins share, with several other factors, an N-terminal 57-amino acid sequence termed the MADS domain, which is responsible for protein dimerization and sequence-specific DNA-binding (Shore and Sharrocks, 1995). The MADS domain is an acronym for the earliest-described members of the protein family: the yeast mating type regulator MCM1; the plant floral determinants Agamous and Deficiens/Apetala3; and the animal protein Serum response factor (SRF) (Black and Olson, 1998).

Phylogenetic analyses have concluded that the MADS protein domain is ancient and originated in a common ancestor of prokaryotes and eukaryotes, based on similarities in primary structure between eukaryotic MADS proteins and the *Escherichia coli* universal stress protein UspA (Mushegian and Koonin, 1996). Current evolutionary models propose that an ancestral MADS-box gene was duplicated prior to the divergence of the plant and animal kingdoms, and these duplicates formed the founders of the two major classes of eukaryotic MADS domain proteins found today: type I and type II MADS domain proteins (Alvarez-Buylla et al., 2000). In higher animals, type I proteins are represented by serum response factor, which contains a conserved SAM domain immediately C-terminal to the MADS domain. The SAM domain functions in homo- and heterodimerization (Ling et al., 1998). Interestingly, despite the ancient evolution of serum response factor proteins, sequenced animal genomes contain only one serum response factor gene member per haploid genome. By contrast, type II MADS domain proteins have diverged significantly to generate additional family members, particularly in plants (Theissen et al., 1996; Becker and Theissen, 2003). In addition to the MADS domain, plant type II proteins also contain a conserved K domain for protein–protein interaction (Yang and Jack, 2004), whereas animal type II proteins acquired a 29-amino acid sequence which was termed the MEF2 domain, based on its inclusion in animal MEF2 proteins (Theissen et al., 1996; Black and Olson, 1998).

The diversity of organisms encoding type I and type II MADS domain proteins indicates that MEF2 proteins arose early in the evolution of life on Earth, and have been retained in the genomes of organisms since that time to

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fulfill functions essential to the development and survival of the organism. Indeed, it appears that all eukaryotic genomes contain at least one member of each of the type I and type II MADS domain families.

II.C. Structure of MEF2 Proteins

A striking feature of the MEF2 family is the retention of the MADS domain and the adjacent MEF2 domain at the N-terminus of all known MEF2 proteins. This is clearly apparent in comparisons of the overall domain structures of mammalian MEF2 proteins with that of *Drosophila* (Fig. 1A). In serum response factor (see Chapter 9.3), by contrast, the MADS domain begins at amino acid 141. Structural studies comparing MEF2 to serum response factor indicate that the presence of the MADS domain at the N-terminus affects DNA-binding and likely accounts for the differential binding sites preferred by MEF2 compared to serum response factor (West et al., 1997; Santelli and Richmond, 2000). The MADS and MEF2 domains have been deeply conserved throughout evolution. For example, within the combined MADS and MEF2 domains, *Drosophila* MEF2 differs from mouse MEF2D at only 9 of 86 amino acid residues (Fig. 1B).

As might be expected from the high sequence conservation within N-termini of MEF2 proteins, critical functions are imparted by the MADS and MEF2 domains. Deletion analyses confirmed that the MADS domain was required, although not sufficient, for DNA-binding. Full DNA-binding by MEF2 also required the presence of the MEF2 domain (Huang et al., 2000). Furthermore, MEF2 factors also dimerize via the MADS and MEF2 domains (Pollock and Treisman, 1991).

To define the residues within MEF2 that contribute to dimerization and DNA-binding, extensive mutagenesis studies were conducted by Molkentin and colleagues. These authors generated a series of 22 point mutants of MEF2C, containing alterations in the sequence of the MADS and MEF2 domains, and assayed each mutant protein for dimerization, DNA-binding and transcriptional activation potential (Molkentin et al., 1996a). These studies identified three critical regions within the MADS domain and one region in the MEF2 domain that were required for DNA binding: amino acids 3–5; 23–24; 30–31; and 68–72 (Molkentin et al., 1996a). In addition, residues 35–50 of the MADS domain were critical for dimerization. Consistent with the *in vitro* mutagenesis studies, randomly-induced point mutants of the *Drosophila Mef2* gene have

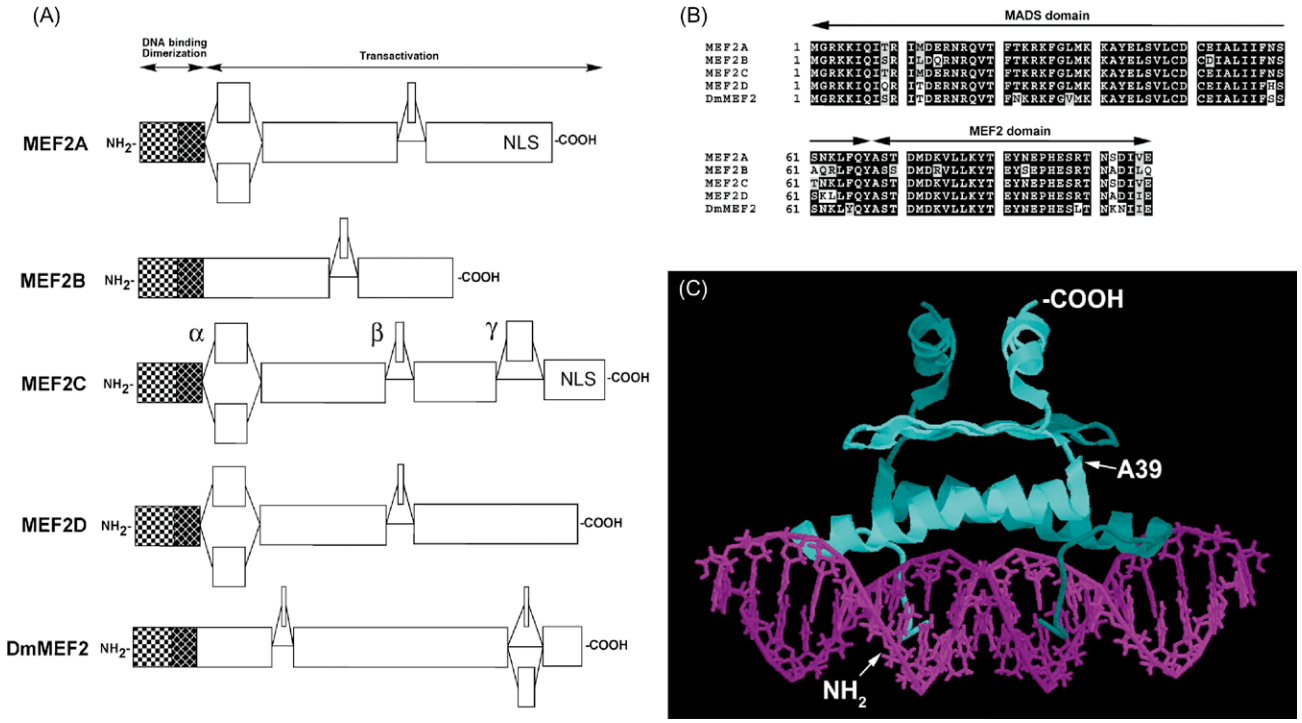

FIGURE 1 Structure of MEF2 factors. (A) Domain structure of the four mammalian MEF2 factors MEF2A–D, and *Drosophila* MEF2. Note that each protein comprises N-terminally located MADS and MEF2 domains (shaded boxes) which function in dimerization and DNA-binding. The C-terminal regions (open boxes) are highly variable. In mammals, variability usually centers around three main regions termed α, β and γ (indicated on MEF2C). (B) Sequence conservation among the MADS and MEF2 domains of murine and *Drosophila* MEF2 proteins. (C) Structure of a human MEF2A dimer complexed with DNA. Amino acids 1–85 are shown. DNA strands are shown in two shades of purple, and the two MEF2A polypeptides are shown in two shades of blue. Amino acid A39 is shown for orientation purposes. Image was created using Protein Explorer and the structure coordinates contained in Accession #1C7U. Huang et al. (2000).

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also established the importance of the MADS domain for MEF2 function *in vivo* (Nguyen et al., 2002). Three point mutants of *Drosophila* MEF2 that affect conserved MADS domain residues ablated MEF2 DNA-binding (Nguyen et al., 2002). Notably, one of the residues affected in these mutants, R24, mimics the biochemical defects of the orthologous mutant mammalian protein (Molkentin et al., 1996a; Nguyen et al., 2002).

More recently, a rigorous evaluation of the MEF2C mutagenesis data has been possible, with the determination by two separate groups of the crystal structure of the N-terminus of MEF2A bound to DNA (Fig. 1C) (Huang et al., 2000; Santelli and Richmond, 2000). The two structures are in close agreement with each other, and also with the published mutagenesis studies from Molkentin and colleagues. The structural analyses show that the N-terminus of MEF2A (amino acids 1–10) forms an extension that contacts the minor groove of DNA, presumably to stabilize protein–DNA interactions, while amino acids 13–36 form an α-helix that contacts DNA at several sites in the major groove (Huang et al., 2000; Santelli and Richmond, 2000). Critical interacting residues are dispersed in the region encompassing amino acids 13–36, but also include amino acids 23–24 and 30–31, which were defined by functional studies. Following the α-helical region, the MADS domain forms two antiparallel β-sheets, comprising approximately amino acids 40–60. In the crystal structure, these motifs are critical contact points for dimerization of MEF2 (Huang et al., 2000; Santelli and Richmond, 2000). These observations are also in agreement with the mutagenesis studies, which showed that residues 35–50 were critical for protein–protein interactions within the dimer (Molkentin et al., 1996a). Finally, the MEF2 domain forms a short α-helical region (amino acids 63–73), which also appears to function in dimerization, since the location of the helix is remote from the DNA, and the structure predicts a contact point between dimerized MEF2A polypeptides. Here, the structural data diverge slightly from those predicted by mutagenesis studies, which suggested that the MEF2 domain mutations affected DNA-binding but not dimerization (Molkentin et al., 1996a). These differences might be explained if the function of the MEF2 domain α-helix residues was to stabilize the dimer after it had formed. *In vitro* dimerization studies might not be sensitive enough to detect a mild destabilization, but this could be reflected in attenuated DNA-binding.

There is also compelling evidence that the MADS and MEF2 domains function critically in the activation of target gene expression. Again, the first evidence in support of this came from the mutagenesis studies, which showed that individual mutation of several different residues scattered throughout the first 86 amino acids did not affect DNA-binding, but had severe effects on transcriptional activation ability (Molkentin et al., 1996a). These observations are consistent with the large number of co-factors that interact with MEF2 through the N-terminal domains, as well as the observation that the phosphorylation of a serine at the junction of the MADS and MEF2 domains is an important post-translational mechanism for MEF2 regulation (Molkentin et al., 1996a; Cox et al., 2003). Phosphorylation and other post-translational modifications of MEF2 will be discussed in detail in Section III of this chapter.

In contrast to the MADS and MEF2 domains, the C-terminal regions of MEF2 proteins are highly-divergent and also highly-variable within a single gene, as a result of regulated RNA splicing. MEF2 primary transcripts are subjected to alternative splicing, skip splicing and cryptic splice site selection, which generates a large number of potential MEF2 isoforms (Black and Olson, 1998; Zhu and Gulick, 2004; Zhu et al., 2005). For *Mef2d*, the alternative splicing of the exon immediately C-terminal to the MEF2 domain is regulated in a tissue-specific manner to give rise to a muscle-specific isoform (Fig. 1A) (Breitbart et al., 1993; Martin et al., 1994), and a similar pattern of alternative splicing of an equivalent domain has been observed for *Mef2a* and *Mef2c* transcripts (Martin et al., 1993; McDermott et al., 1993; Zhu and Gulick, 2004). The *Drosophila Mef2* primary transcript is also subject to regulated splicing (Taylor et al., 1995).

Recent studies from Gulick and colleagues have categorized the different protein domains resulting from regulated splicing of mammalian *Mef2c* transcripts as α, β and γ (Fig. 1A) (Zhu and Gulick, 2004). *Mef2a* and *Mef2d* transcripts also show alternative splicing of the α and β regions, and constitutively include sequences encoding the γ domain (Zhu et al., 2005). The β domain enhances transcriptional activation by the parent MEF2 molecule, and is also preferentially-included in brain and muscle transcripts. In contrast, the γ domain is alternatively spliced in *Mef2c*, and acts as a phosphorylation-dependent transcriptional repressor (Zhu et al., 2005). These studies indicate that MEF2 function is modulated by alternative RNA splicing. Given the recent observation that alternate splicing of *Mef2b* transcripts is altered in *Mef2c*-null hearts (Vong et al., 2006), pathological conditions might significantly affect the patterns of *Mef2* transcript splicing, and thus alter MEF2 function.

In addition to transcriptional activation functions, the C-termini of MEF2A, MEF2C and MEF2D each contain nuclear localization signals (Fig. 1A), which are critical to *in vivo* function (Yu, 1996; Borghi et al., 2001). Furthermore, a *Drosophila Mef2* mutant allele encodes a C-terminally truncated isoform that does not localize to the nucleus, suggesting that the location of nuclear localization signals is generally conserved across MEF2 proteins (Ranganayakulu et al., 1995). Furthermore, and as discussed in the next section, the C-terminal regions of MEF2 proteins contain potent transcriptional activation domains,

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and are also important targets for phosphorylation and other post-translational modifications that regulate MEF2 function.

III. REGULATION OF MEF2 ACTIVITY BY POST-TRANSLATIONAL MODIFICATION

III.A. MEF2 Functions as a Transcriptional Co-Factor

MEF2 transcription factors interact with a diverse array of co-factors that modulate MEF2 activity. MEF2 can function either as an activator or as a repressor, depending on co-factor interactions, and several MEF2 co-factors facilitate the ability of MEF2 to respond to intracellular signaling. MEF2 function as a transcriptional co-factor was first described from studies in skeletal muscle, where MEF2 proteins were shown to function as essential co-factors for the myogenic bHLH proteins, including MyoD and myogenin (Molkentin et al., 1995; Ornatsky et al., 1997; Black et al., 1998). Myogenic bHLH proteins have the remarkable ability to convert nonmuscle cells to muscle cells in culture, and it was observed that this activity was dependent on interaction with MEF2 (Molkentin et al., 1995; Ornatsky et al., 1997; Black et al., 1998). MEF2 and MyoD physically associate, and their binding sites are frequently coordinately positioned in the enhancers and promoters of muscle-specific genes (Molkentin et al., 1995; Fickett, 1996; Black et al., 1998). The interaction of MyoD and MEF2 occurs through the DNA-binding motifs of each factor, the bHLH domain on MyoD and the MADS and MEF2 domains of MEF2, raising the possibility that MEF2 factors may interact with a wide array of bHLH proteins.

Indeed, MEF2 proteins interact with several other bHLH proteins in diverse contexts. MEF2 forms a complex and potently activates transcription of target genes in cooperation with the neural bHLH protein mammalian achaete-scute homolog 1 (MASH1), and this interaction is also dependent on the MADS and MEF2 domains (Black et al., 1996; Mao and Nadal-Ginard, 1996). In the heart, MEF2C cooperatively activates transcription of the *Nppa* gene with the bHLH proteins HAND1 and HAND2, which like MEF2, are essential regulators of cardiac development (Srivastava et al., 1995; Zang et al., 2004; Morin et al., 2005).

GATA4 is another cardiac-enriched transcription factor that interacts with MEF2 to activate the *Nppa* promoter (Morin et al., 2000) (see Chapter 9.2). Given the broad overlap in the expression of GATA and MEF2 transcription factors and the prevalence of GATA and MEF2 sites in cardiac promoters, these members of these two families of transcription factors may participate in the co-activation of numerous other genes in the heart (Vanpoucke et al., 2004). The homeodomain protein Pitx2 also interacts with MEF2A to activate the *Nppa* promoter synergistically (Toro et al., 2004). This interaction requires MEF2-binding to the promoter, suggesting that MEF2 may serve as a platform for Pitx2-binding, and that direct interaction of Pitx2 with a *cis*-acting element in the promoter may not be required (Toro et al., 2004).

The majority of MEF2-interacting transcription factors discussed above contact MEF2 through the MADS and MEF2 domain. The transcription factor TEF-1 is an example of a MEF2 co-factor that does not bind to the MADS domain. Rather, TEF-1 interacts with motifs present near the C-terminus of MEF2C (Maeda et al., 2002). TEF-1 usually binds to MCAT elements, which are present in the promoters and enhancers of numerous skeletal and cardiac muscle genes (Mar and Ordahl, 1990). Many of these gene promoters also contain conserved MEF2 sites, which suggest the possibility that MEF2C and TEF-1 may co-regulate multiple genes involved in cardiac development and differentiation. In addition, it has been observed that TEF-1 can bind directly to MEF2 sites or to MEF2-like AT-rich elements, suggesting a further interplay between MEF2 and TEF-1 during muscle development, possibly through competition for shared binding elements (Karasseva et al., 2003).

Thyroid hormone receptor (TR) is another MEF2 co-factor that interacts with MEF2 in the heart, and this interaction is facilitated by p300/CBP, which is thought to bridge the two factors and promote transcriptional activation (De Luca et al., 2003). TR and MEF2 interaction has been shown to be important for the activation of the α-MHC gene via closely-positioned binding sites for the two factors in the proximal promoter region (Lee et al., 1997). The interaction of MEF2 and MyoD in skeletal muscle is also facilitated by p300 (Sartorelli et al., 1997). In addition to acetylation of histones and subsequent chromatin relaxation, p300 also directly acetylates MEF2, which promotes MEF2 transactivation, probably through negative regulation of sumoylation (Ma et al., 2005; Zhao et al., 2005a), which will be discussed later in this chapter. The cooperative transcriptional activation of the α-MHC promoter by TR and MEF2 is attenuated by the action of Jumonji/Jarid2, which is also a direct MEF2 partner (Kim et al., 2005). Jumonji, a histone demethylase, directly interacts with the MADS domain of MEF2A to negatively regulate MEF2-dependent transcription (Kim et al., 2005). Jumonji interaction with MEF2A probably blocks TR interaction with the MADS domain, and represses transcription via a direct effect on histones.

In addition to MEF2 co-factors that interact with DNA and MEF2, there are a number of tissue-specific and ubiquitous proteins that modulate MEF2 activity solely through protein–protein interactions. One such group of MEF2-interacting factors includes the SAP domain proteins myocardin and MASTR. Myocardin was first identified as a potent transcriptional activation partner for

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serum response factor (see Chapter 9.3), a MADS domain protein that is closely related to MEF2, in cardiac and smooth muscle (Wang et al., 2003b; Yoshida et al., 2003). Myocardin itself does not bind DNA, but uses DNA-bound serum response factor as a platform to interact with chromatin through protein–protein interactions. Once bound to serum response factor, myocardin very potently activates transcription (Wang et al., 2003b). One molecule of myocardin interacts with each serum response factor dimer bound to a CArG box, and myocardin itself probably dimerizes to bridge two CArG elements (Wang et al., 2003b; Yoshida et al., 2003). A longer form of myocardin, generated from a distinct splicing event can interact with either serum response factor or MEF2 (Creemers et al., 2006). The MEF2 interaction domain in the myocardin long-form comprises a short amino acid sequence at the N-terminus that is distinct from the N-terminus of the short-form of myocardin, which facilitates serum response factor interaction (Creemers et al., 2006). The unique N-terminus of the myocardin long-form directly interacts with MEF2. Interestingly, however, the myocardin long-form still retains the sequences necessary to facilitate interaction with serum response factor, such that this isoform of myocardin can interact with either serum response factor or MEF2 (Creemers et al., 2006).

Olson and colleagues used the unique N-terminal sequence of the myocardin long-form to identify another SAP domain-containing protein that also contains the MEF2 interacting sequence (Creemers et al., 2006). This myocardin homolog was named MASTR (MEF2-associated SAP domain transcriptional regulator). Unlike the myocardin long-form, MASTR only contains sequences sufficient to interact with MEF2 and not with serum response factor (Creemers et al., 2006). Thus, the myocardin family includes: the myocardin short-form, which only interacts with serum response factor; MASTR, which only interacts with MEF2; and the myocardin long-form, which can interact with either MEF2 or serum response factor. The myocardin long- and short-forms contain sequences that should allow dimerization between the two isoforms, which raises the intriguing possibility that myocardin heterodimers, containing long- and short-forms, might bridge serum response factor and MEF2 binding sites. Since numerous promoters contain binding elements for each of these classes of MADS domain transcription factors, myocardin members may bridge the sites and allow synergistic activation mediated by MEF2 and serum response factor. This possible relationship between serum response factor and MEF2 through myocardin may be further facilitated by the presence of binding sites that can be bound by either MEF2 or serum response factor (L'Honore et al., 2007). These composite SRF/MEF2 *cis*-elements provide additional targets for myocardin and MASTR regulation of cardiac genes. To date, no specific genes have been identified as myocardin targets through the bridging of MEF2 and serum response factor bound to their respective cognate binding sites, but it seems likely that this mechanism will function in the heart and other tissues. If this notion is correct, it would provide an additional mode for MEF2 to serve as a transcriptional switch through the assembly of a multi-protein complex.

III.B. Chromatin Remodeling by MEF2 through Interaction with Histone Deacetylases

It is now becoming increasingly appreciated that binding sites for regulatory factors must be accessible in the context of the overall chromatin structure of the cell in order to be recognized, and for gene expression to be controlled. Along these lines, it now appears that a major function of MEF2 is to control the balance between chromatin acetylation and deacetylation, and thereby regulate the relative accessibility of promoters and enhancers to the transcriptional machinery (Fig. 2). Accordingly, MEF2 factors interact with multiple histone acetylases and deacetylases. Most notably, MEF2 forms a complex with class II histone deacetylases (HDACs), which include HDACs 4, 5, 6, 7 and 9 (McKinsey et al., 2001a) (see Chapter 10.2). Interaction with class II HDACs occurs through the MADS domain at the N-terminus of MEF2 (Lu et al., 2000b; Dressel et al., 2001; Zhang et al., 2001a). Similarly, a conserved N-terminal domain in HDAC dictates interaction with MEF2 (Wang et al., 1999; Lemercier et al., 2000; Dressel et al., 2001). MEF2-HDAC complexes repress transcription by deacetylating histones, resulting in chromatin condensation and reduced accessibility of core transcriptional machinery to promoter and enhancer regions of MEF2 target genes (Lu et al., 2000b; Kao et al., 2001; McKinsey et al., 2001a). MEF2 also interacts with several histone acetyltransferases, including p300/CBP and SIRT1, which likely serve to balance the repressive effects of HDAC on MEF2 and allow MEF2 to function as a transcriptional switch (Sartorelli et al., 1999; Ma et al., 2005; Zhao et al., 2005a; Stankovic-Valentin et al., 2007).

Class II HDACs are important regulators of transcription in the developing and postnatal heart that help to regulate the hypertrophic response (Zhang et al., 2002; Olson et al., 2006). Normal growth of the myocardium requires large amounts of structural and other regulatory proteins to be synthesized as cells enlarge, but excessive enlargement of the heart can result in pathologic hypertrophy, which ultimately can lead to heart failure (Olson et al., 2006). Thus, HDACs serve as a kind of regulated braking mechanism, keeping the MEF2-dependent transcriptional response in check until signals that stimulate myocardial growth are received. Hypertrophic induction results in signal-dependent export of HDACs from the nucleus, which results in chromatin relaxation due to increased histone

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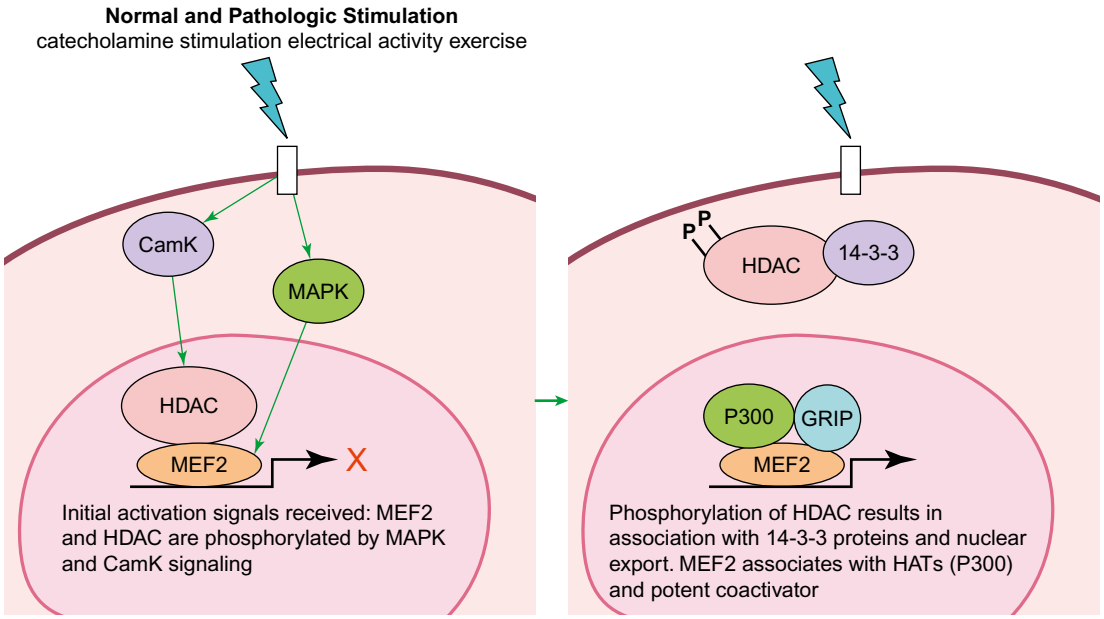

FIGURE 2 MEF2 functions as a signal-dependent transcriptional switch. MEF2 functions as a repressor by recruiting class II HDACs to promoter and enhancer regions of target genes. In response to a variety of developmental and pathological signals, CamK and MAPK signaling pathways are activated. These signals result in the phosphorylation of MEF2 and HDACs. HDAC phosphorylation by CamK results in exposure of a nuclear export signal at the C-terminus, interaction with 14-3-3 proteins, and export from the nucleus. MEF2 phosphorylation and HDAC dissociation result in recruitment of HATs and co-activator molecules, such as GRIP-1, and the conversion of MEF2 to an activator complex.

acetylation and subsequent MEF2-dependent transcription (McKinsey et al., 2000a; Kao et al., 2001; McKinsey et al., 2001a,b; Olson et al., 2006).

Intriguingly, the deacetylase activity of class II HDACs is not required for interaction with MEF2, nor is this activity required for transcriptional repression by MEF2-HDAC (Lemercier et al., 2000; Zhang et al., 2001b; Chan et al., 2003). The dispensability of deacetylase activity is consistent with the observations that MITR (MEF2-interacting transcriptional repressor), an HDAC homolog lacking the deacetylase domain, also interacts with MEF2 and facilitates strong transcriptional repression, as its name implies (Youn et al., 2000; Zhang et al., 2001b). This may occur because class II HDACs and MITR have the ability to recruit the potent co-repressor protein CtBP (Dressel et al., 2001; Zhang et al., 2001a). CtBP co-repressors repress transcription via recruitment of HDACs (Bertos et al., 2001). CtBP physically associates with the N-terminus of HDAC4, HDAC5 and the HDAC homolog MITR, which interact with MEF2 to repress its activity (Zhang et al., 2001a). The deacetylase activity-independent repression might also result from the observation that HDACs can multimerize, allowing deactylase-defective HDACs to recruit other HDACs that possess full enzymatic activity, although this may not explain the strong transcriptional repression conferred to MEF2 by MITR (Youn et al., 2000; Zhang et al., 2001b). Alternatively, a crucial function of class II HDACs may be to help control the balance of acetylation and sumoylation of MEF2. These two post-translational modifications have mutually-exclusive and opposing functions in promoting MEF2-dependent activation and repression, respectively (Zhao et al., 2005a; Gregoire et al., 2006; Shalizi et al., 2006; Stankovic-Valentin et al., 2007). In addition to its role in deacetylation of histones, HDAC4 also inhibits MEF2-dependent transcription by promoting sumoylation of MEF2 (Zhao et al., 2005a; Gregoire et al., 2006; Stankovic-Valentin et al., 2007).

As noted above, it was recently discovered that MEF2 proteins are modified by sumoylation (Gregoire et al., 2006; Kang et al., 2006; Riquelme et al., 2006; Shalizi et al., 2006). Sumoylation is the process by which a protein moiety, SUMO, is covalently added to proteins by the activity of SUMO-conjugating enzymes (Gill, 2005). Addition of SUMO-1 to the C-terminus of MEF2 modifies MEF2 to a repressor form, which has been demonstrated for MEF2A, MEF2C and MEF2D (Gregoire and Yang, 2005; Zhao et al., 2005a; Kang et al., 2006; Shalizi et al., 2006). Interestingly, SUMO addition to MEF2 occurs at a lysine residue in the C-terminal activation domain and is controlled by MAPK phosphorylation at a nearby serine residue for MEF2A, MEF2C and MEF2D (Fig. 3) (Gregoire et al., 2006; Kang et al., 2006; Shalizi et al., 2006). In each case, dephosphorylation of MEF2 promotes a switch from acetylation to sumoylation at the neighboring lysine and the conversion of MEF2 from an activator to a repressor (Gregoire et al., 2006; Kang et al., 2006; Shalizi et al., 2006). The dephosphorylation event is

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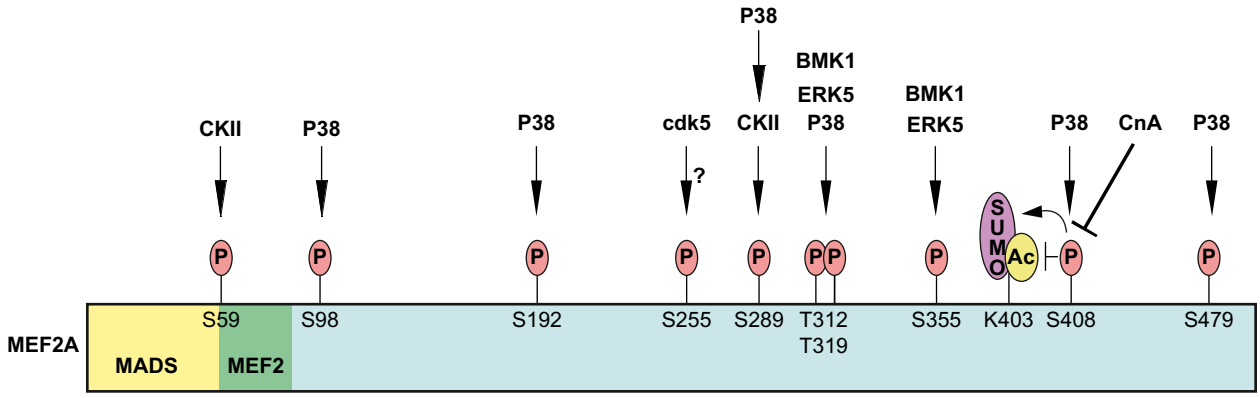

FIGURE 3 MEF2 proteins are extensively modified post-translationally. A schematic of human MEF2A shows sites of phosphorylation (red ovals), sumoylation (pink oval) and acetylation (yellow oval). Signaling pathways that are known or thought to modify MEF2 are noted above and the amino acid residues are noted below. The MADS and MEF2 domains are depicted. Differential modification of lysine 403 by acetylation and sumoylation is controlled by phosphorylation and dephosphorylation by p38 and calcineurin at the neighboring serine 408. Note that similar (but not identical) post-translational modifications depicted here for hMEF2A also occur on other MEF2 isoforms.

controlled by calcineurin, which serves as a link between calcium-mediated hypertrophic signaling and post-translational modification of MEF2 by sumoylation (Flavell et al., 2006; Shalizi et al., 2006).

In contrast to sumoylation, direct acetylation of MEF2C at the C-terminus by CBP promotes MEF2 activity, as noted earlier in this chapter (Sartorelli et al., 1997). The balance between sumoylation and acetylation of MEF2 is also controlled directly by HDACs themselves. HDAC4 has been shown to promote SUMO addition to MEF2, providing a dual mechanism for repression of MEF2 activity by class II HDACs through deacetylation of chromatin, and further through addition of SUMO to MEF2 (Zhao et al., 2005a; Gregoire et al., 2006). This may partially explain why the enzymatic activity of HDACs is not required for repression of MEF2, since the catalytic domain of HDAC4 is not required for sumoylation of MEF2 (Zhao et al., 2005a). Interestingly, HDAC4 is also regulated by sumoylation. However, sumoylation of HDAC4 inhibits its ability to promote SUMO addition to MEF2, which provides a post-translational negative-feedback mechanism for control of MEF2 sumoylation and repression (Zhao et al., 2005a; Gregoire et al., 2006).

It was previously believed that only class II HDACs interacted with MEF2, but several reports have now demonstrated that class I HDACs also interact with MEF2, and that these interactions play an important role in the heart (Montgomery et al., 2007; Trivedi et al., 2007). The class I HDAC, HDAC3, was shown recently to interact with and supress the transcriptional activity of MEF2 (Gregoire et al., 2007). The nature of the interaction between class I HDACs and MEF2 is not as well-characterized as the interaction with class II HDACs, so it remains unclear which residues within the MEF2 MADS domain make direct contact with the class I HDAC (Gregoire et al., 2007). Structural studies should resolve how the interactions of these two broad classes of HDACs with MEF2 result in different biological outputs.

III.C. MEF2 Functions as a Signal-Dependent Transcriptional Switch

The interaction of MEF2 with HDACs underscores the function of MEF2 proteins as both positive and negative regulators of transcription. Prior to those groundbreaking observations, MEF2 proteins were generally thought of only as transcriptional activators that functioned through protein–protein interactions with other transcription factors containing more potent activation domains (Molkentin and Olson, 1996; Black and Olson, 1998). The notion that MEF2 functions as both a repressor and an activator, depending on the gene, cell type and cellular differentiation state led to the idea that MEF2 serves as a switch capable of interpreting distinct signals into opposing transcriptional outputs. Therefore, it has been important to define what dictates whether MEF2 functions as an activator or a repressor. Over the last decade, numerous intracellular signaling pathways have been identified to interact with MEF2 and class II HDACs. Not surprisingly, MEF2 and HDAC proteins are each regulated by their phosphorylation state (McKinsey et al., 2001a, 2002).

Class II HDACs are phosphorylated in response to a variety of extracellular signals, including electrical activity, pressure, adrenergic signaling and other normal developmental and postnatal cues. These signals result in an increase in the concentration of Ca^{2+} in the cytoplasm, which activates the phosphatase calcineurin and stimulates the activity of calcium/calmodulin-dependent kinases (CaMK) I, II and VI (Lu et al., 2000a; Kao et al., 2001; McKinsey et al., 2001b; Little et al., 2007). Phosphorylation of class II HDACs by CaMK occurs on

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two residues near the N-terminus of the HDAC protein (McKinsey et al., 2000a,b; Kao et al., 2001). CaMK phosphorylation of HDAC facilitates interaction with 14-3-3 proteins, which activates a nuclear export sequence at the C-terminus (McKinsey et al., 2000b; Choi et al., 2001; McKinsey et al., 2001b; Wang and Yang, 2001). HDAC nuclear export, in turn, results in MEF2 activation of gene expression in the heart and other MEF2-dependent tissues (Fig. 2). In addition, SIK1 kinase phosphorylates class II HDACs, which affects MEF2-dependent transcription through dissociation of MEF2 from HDAC (Berdeaux et al., 2007).

MEF2 factors themselves are extensively phosphorylated in response to a host of intracellular and extracellular cues (Fig. 3). The p38, BMK1/ERK5 and ERK1 mitogen-activated protein kinases (MAPK) each play a role in MEF2 regulation through phosphorylation. BMK1/ERK5 signaling results in the phosphorylation of MEF2C at serine-387 in the C-terminal activation domain in response to a variety of extracellular signals, including adrenergic signaling and pressure overload in the myocardium (Kato et al., 1997; Yang et al., 1998; Yan et al., 1999; Nadruz et al., 2003). Each of these cues stimulates the nuclear localization of BMK1/ERK5, which leads to phosphorylation and activation of MEF2 (Kato et al., 2000; Yan et al., 2001). Although these studies investigated BMK1/ERK5 signaling in the postnatal heart, it is also quite likely that BMK1/ERK5 signaling regulates MEF2 phosphorylation during cardiac development, since all of these signals lead to MEF2-dependent *c-jun* transcription, which functions in numerous developmental contexts (Kato et al., 1997; Marinissen et al., 1999; Nadruz et al., 2003).

The p38 MAPK signaling pathway also plays a fundamentally important role in the post-translational modification of MEF2 in myocytes (Han and Molkentin, 2000). Signaling by p38 results in phosphorylation of MEF2A and MEF2C, but not MEF2B or MEF2D (Han et al., 1997; Ornatsky et al., 1999; Zhao et al., 1999; Chang et al., 2002). p38 phosphorylation promotes the role of MEF2 as a transcriptional activator in response to normal developmental and postnatal hypertrophic growth of the heart, as well as to pathologic hypertrophic cues (Han et al., 1997; Kolodziejczyk et al., 1999; Ornatsky et al., 1999; Zhao et al., 1999; Han and Molkentin, 2000; Cox et al., 2003). Other studies have shown that retinoic acid (RA) signaling during myocardial development results in the phosphorylation of MEF2 via p38 MAPK (Ren et al., 2007), which potentially links normal growth and development of the myocardium via RA signaling to MEF2 (Tran and Sucov, 1998; Lavine et al., 2005). Casein kinase II (CKII) results in phosphorylation of a conserved serine (S59) found at the junction of the MADS and MEF2 domains in MEF2A and MEF2C (Molkentin et al., 1996c; Cox et al., 2003). Work from McDermott and colleagues showed that CKII also results in the direct phosphorylation of MEF2A at serine 289 in response to p38 MAPK signaling (Fig. 3) (Cox et al., 2003).

An interesting study of MEF2 function in dominant induction of skeletal myogenesis in culture showed that RAF kinase inhibits MyoD-induced conversion of fibroblasts into muscle cells by blocking MEF2 nuclear localization (Winter and Arnold, 2000). These studies were the first to suggest a regulated nuclear localization of MEF2 proteins themselves, which may have important implications if MEF2 is shuttled between the nucleus and cytoplasm during the development of the heart and other tissues.

Cyclins and cyclin-dependent kinase pathways have also been shown to regulate MEF2 activity and to provide a link between the cell-cycle and MEF2-dependent transcription. For example, cdk5 phosphorylates MEF2C in neurons, supporting the notion that a cell-cycle-dependent signaling event functions via post-translational modification of MEF2C in neurons (Gong et al., 2003; Tang et al., 2005; Smith et al., 2006). The role of cdk phosphorylation of MEF2 in the heart has not been examined, but it is likely that MEF2 plays a role in cell-cycle control along with GATA4, which is known to regulate cardiac cell-cycle, and to interact with MEF2 and other partners downstream of these signaling pathways (Morin et al., 2000; Vanpoucke et al., 2004; Zeisberg et al., 2005; Xin et al., 2006).

MEF2 factors themselves have only weak inherent transcriptional activation potential (Molkentin et al., 1996a,b). This is also the case for several of the many MEF2 co-factors that have been described to date, such as GATA4. However, MEF2 proteins and their co-factors are sufficient to direct extremely robust activation of many genes and reporter genes, both *in vivo* and in reporter assays in cell culture. A likely mechanism for how MEF2 is able to drive strong transcriptional activation is through interaction with potent transcriptional co-activators, such as GRIP-1, which belongs to the p160 steroid receptor co-activator (SRC) family of transcriptional co-activator proteins (Chen et al., 2000; Leo and Chen, 2000; Lazaro et al., 2002; Xu and Li, 2003; Liu et al., 2004). Signaling downstream of D cyclins and cdk4 activity blocks muscle-differentiation by disrupting the interaction between MEF2C and GRIP-1 (Lazaro et al., 2002).

Interactions with transcriptional co-activators and co-repressors provide another mechanism for MEF2 to serve as a transcriptional switch, repressing transcription in some contexts, while activating it in others. In this regard, the interaction between MEF2 and GRIP-1 is also targeted by the TGFβ signaling pathway (Liu et al., 2004). TGFβ signaling is transmitted intracellularly by Smads transcription factors, and MEF2 interacts with several different members of the Smads family, including Smads 2, 3 and 4 (Quinn et al., 2001; Derynck and Zhang, 2003; Liu et al., 2004). Smads 2 and 4 interact with MEF2 to influence transcription positively in skeletal muscle by promoting

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interaction between MEF2 and MyoD (Quinn et al., 2001). Presumably, TGFβ or BMP signaling influences MEF2 activity in the heart through interactions with Smads as well, although this has not been demonstrated. In contrast, Smad3 is an inhibitory Smad and functions as a negative regulator of transcription (Liu et al., 2004). Smad3 associates with MEF2 and disrupts MEF2 association with GRIP-1, thereby repressing transcription by targeting the interaction of MEF2 and one of its co-activators (Liu et al., 2004).

MAML1 (mastermind like 1) is also a potent transcriptional co-activator that interacts with MEF2 (Shen et al., 2006). MAML factors belong to a family of co-activator proteins that are required for Notch signaling. Notch signaling inhibits skeletal muscle differentiation and the muscle-inducing activity of myogenic bHLH proteins (Kopan et al., 1994; Kuroda et al., 1999; Wilson-Rawls et al., 1999; Shen et al., 2006). Activated Notch interacts directly with MEF2C via a specific splice isoform of MEF2C found in muscle lineages (Wilson-Rawls et al., 1999). Thus, it is clear that this pathway interacts with MEF2 via Notch itself and via the Notch co-activator MAML1 to inhibit skeletal myogenesis (Wilson-Rawls et al., 1999; Shen et al., 2006). The presence of the muscle-specific exon has not been examined in cardiac muscle, so it is not known whether Notch interacts directly with MEF2C in the heart.

In addition to direct interactions with co-activator molecules, MEF2 also interacts with a number of co-repressor proteins. For example, CABIN1/CAIN is a potent transcriptional co-repressor protein that binds to calcineurin and interacts directly with MEF2 (Esau et al., 2001; Han et al., 2003). Interaction of CABIN1 with MEF2 blocks the activation of MEF2 by BMK1/ERK5 MAP kinase signaling (Kasler et al., 2000). CABIN1 further represses MEF2 by recruitment of mSIN3 and associated HDACs, and CABIN1 also recruits the histone methyltransferase SUV39H1 to the complex (Youn and Liu, 2000; Jang et al., 2007). Chen and colleagues solved the crystal structure of the MADS domain of MEF2B in a complex with CABIN1 (Han et al., 2003). The structure showed that CABIN1 contacts the MADS domain in a similar, but not identical, fashion to class II HDACs, raising the possibility that the binding of HDAC and CABIN1 to MEF2 might be mutually exclusive (Han et al., 2003, 2005). The discrete contacts of each of these MEF2 co-regulators suggest that they likely affect MEF2 conformation differently, but this remains to be determined.

In general, it is clear that the major function of MEF2 is to respond to intracellular signaling pathways and to respond to those signaling cues through co-factor interactions (Figs 2; 3). In response to growth and differentiation signals, MEF2 proteins are modified post-translationally, which is primarily via phosphorylation but also includes acetylation and sumoylation (Fig. 3). These modifications influence whether MEF2 interacts with positive transcriptional co-factors, such as SRC family co-activators, myocardin proteins and histone acetyltransferases, or co-factors that repress transcription, including class II HDACs, MAML, CABIN1 and Jumonji proteins (Czubryt and Olson, 2004; Backs and Olson, 2006; Liu and Olson, 2006). In addition, MEF2 interacts with multiple other transcription factors, which provides additional combinatorial complexity and allows for more precise co-factor recruitment. It is likely that additional MEF2 co-factors will continue to be identified, but the general role for MEF2 as a nodal point for balancing growth and differentiation signals through post-translational modification and co-factor interaction is now well-established.

IV. *MEF2* GENE FUNCTION IN THE HEART AND OTHER TISSUES

IV.A. MEF2 Proteins are Expressed in Multiple Lineages During Development and in Adulthood

Despite the initial identification of MEF2 proteins as factors that are bound to skeletal muscle gene promoters, early studies suggested that MEF2-binding activity was present in a broad tissue distribution (Olson et al., 1995). Indeed, *Mef2d* transcripts are detected in many adult tissues, although the muscle-specific splice variant of *Mef2d* is expressed only in adult muscles (Breitbart et al., 1993; Martin et al., 1994). Furthermore, *Xenopus Mef2* genes are broadly-expressed in the adult frog (Chambers et al., 1992). By contrast, *in situ* hybridization studies performed on vertebrate embryos indicate that expression of *Mef2* genes is indeed predominantly muscle-specific at earlier stages of development (Edmondson et al., 1994). *Mef2* gene expression in frogs and fish is restricted to muscle lineages in the embryo (Chambers et al., 1992; Ticho et al., 1996), and in the mouse embryo, *Mef2a*, *Mef2c* and *Mef2d* expression is largely restricted to skeletal and cardiac muscle early in development (Edmondson et al., 1994). Similarly, mouse *Mef2b* is a marker of early myogenic lineages (Molkentin et al., 1996b).

Nevertheless, expression of *Mef2* genes was also detected in the developing nervous system of mice, suggesting that it might also play a role in neuronal development (Leifer et al., 1994; Ikeshima et al., 1995; Lyons et al., 1995; Lin et al., 1996b). In addition, specific functions for MEF2 have been described more recently in specialized cells of the blood and other tissues of mesodermal and ectodermal origin. MEF2 is an activator of cell death in T-lymphocytes via direct transcriptional activation of the steroid receptor gene *Nur77* (Youn et al., 1999; Youn and Liu, 2000; Liu et al., 2001a). Similarly, immunoglobulin gene expression in B-cells is regulated by MEF2 function (Rao et al., 1998; Satyaraj and Storb, 1998; Wallin

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et al., 1999). In addition, MEF2C is an essential regulator of B-cell proliferation in response to B-cell receptor signaling, which results in direct phosphorylation of MEF2C via p38 MAPK (Khiem et al., 2008; Wilker et al., 2008). Furthermore, *Mef2c* and *Mef2d* are expressed in chondrocytes during bone formation in the embryo (Arnold et al., 2007), and *Mef2c* is highly-expressed in developing neural crest cells in the mouse and fish (Miller et al., 2007; Verzi et al., 2007). Based on these studies, it is clear that although MEF2 is not restricted to muscle lineages, muscle cells in the embryo are a major location of *Mef2* gene expression. Later in vertebrate development, however, transcription of *Mef2* genes is detected in a number of additional tissues and organs, although in many cases the precise cells that express MEF2 within these organs have yet to be defined. In *Drosophila*, embryonic expression of the single *Mef2* gene is restricted to the developing mesodermal tissues, including the cardiac tube (Lilly et al., 1994; Nguyen et al., 1994). Later in development, there is accumulation of MEF2 protein in portions of the *Drosophila* central nervous system (Schulz et al., 1996).

In summary, although they were initially identified as muscle-enriched binding factors, it is now clear that MEF2 proteins function in many tissues during development and in adulthood, including all muscle lineages, neuronal cells, blood cells, neural crest, bone, chondrocytes and vascular endothelium. Direct transcriptional targets of MEF2 have been identified in each of these tissues and, as discussed below, gene knockout strategies and targeted ablation of MEF2 function have begun to define the roles of *Mef2* in many of these cell types more precisely.

IV.B. Genetic Analyses of Mef2 Gene Function

To determine the function of MEF2 in tissues where it is expressed, numerous genetic studies have been performed in flies, mice and fish. The fact that vertebrate genomes have four distinct *Mef2* genes makes defining the precise function of MEF2 complicated in higher animals, due to issues of redundancy and overlapping expression. As noted earlier in this chapter, *Drosophila* has only a single *Mef2* gene, which has made genetic analyses of *Mef2* function highly tractable in that organism. Inactivation of the *Drosophila Mef2* gene results in a severe failure in muscle differentiation for cardiac, skeletal and visceral mesoderm, even though precursors for each of these muscle lineages are normally specified in mutant embryos (Fig. 4) (Bour et al., 1995; Lilly et al., 1995; Ranganayakulu et al., 1995). In *Mef2* mutants, the expression of a large number of muscle structural genes was essentially absent. These potential target genes include the single Myosin heavy-chain gene, *Actin57B*, *Myosin light chain-2*, *Myosin light chain-alkali* and *Troponin I*, and MEF2 binding sites critical for the expression of several of these genes have been described (Bour et al., 1995; Lilly et al., 1995; Ranganayakulu et al., 1995; Kelly et al., 2002; Marin et al., 2004). Interestingly, expression of *Tropomyosin 2* in *Mef2* mutants was reduced in skeletal and visceral muscles, but was relatively normal in the heart (Lin et al., 1996a). Similarly, expression of the β-tubulin gene *βTub60D* was strongly reduced in the skeletal muscles of *Mef2* mutants, but was expressed normally in the cardiac tube (Damm et al., 1998). These two examples emphasize that transcriptional programs can differ significantly between different muscle types in the embryo. Nevertheless, the *Drosophila* studies represent the first example of whole-organism inactivation of MEF2 function and its developmental consequences. These studies remain the clearest examples of MEF2 function and its requirement for the differentiation of muscle lineages.

To define the function of vertebrate *Mef2* genes in cardiac development, a variety of approaches have been employed. In mice, global knockouts of *Mef2c* and *Mef2a* have been performed and have shown that mice lacking these genes have severe defects in cardiac development and function (Lin et al., 1997; Naya et al., 2002). By contrast, a recent publication indicated that homozygotes for a mutation in *Mef2d* are phenotypically normal (Arnold et al., 2007). Targeted inactivation of *Mef2c* in mice results in death by embryonic day (E) 10.5, and mutant mice exhibit severe defects in cardiac development (Lin et al., 1997). Affected embryos display pericardial edema, a

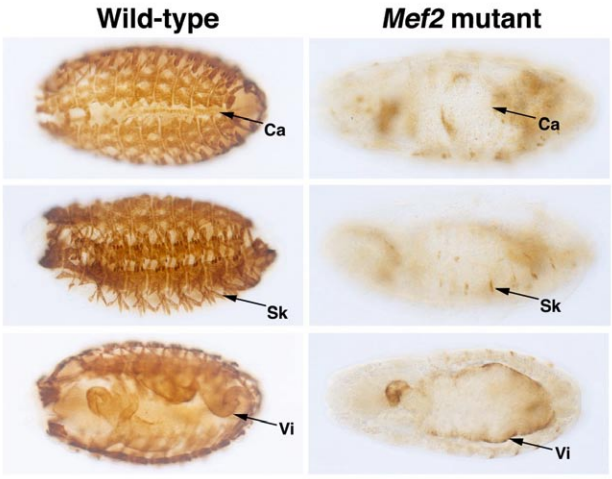

FIGURE 4 Requirement for MEF2 function for cardiac development in *Drosophila*. Muscle tissue can be visualized in mature embryos via immunohistochemical staining for myosin heavy-chain (MHC) protein. Left column: In wild-type embryos, cardiac muscle (Ca) forms at the dorsal midline; skeletal muscles (Sk) are apparent in dorsal and lateral views; and visceral muscle (Vi) showing MHC accumulation can be observed in a ventral view. Right column: in Mef2-null mutants, there is no detectable MHC accumulation in cardiac muscle, only a handful of skeletal muscle cells accumulate MHC, and the visceral muscle accumulates low levels of MHC. In addition, morphogenesis of the mutant gut is abnormal, with the gut predominantly comprising a single lumen.

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failure of cardiac looping, a reduced heart rate and the lack of a well-defined right ventricle (Fig. 5) (Lin et al., 1997). These morphological defects can now be reinterpreted in the context of the second heart field, which has been shown to contribute predominantly to the outflow tract and right ventricle (Kelly and Buckingham, 2002; Black, 2007). Clearly, *Mef2c* mutants show profound defects in structures derived from the second heart field (see Chapter 2.2), although second heart field cells do contribute to the common ventricular chamber in *Mef2c*-null mice (Fig. 5C,D) (Verzi et al., 2005). In addition to the morphological defects, *Mef2c* mutants displayed a downregulation of a number of cardiac muscle structural genes, including *Nppa*, *cardiac α-actin*, *α-myosin heavy chain*, *myosin light chain (MLC) 1A*, *angiopoietin 1* and *Vegf* (Lin et al., 1997; Bi et al., 1999). Note that relatively few of the downregulated genes were those previously demonstrated to be MEF2 targets (Molkentin and Markham, 1993; Lee et al., 1997). This may result from indirect effects of MEF2C on target genes, either acting via an intermediate transcriptional regulator or by modulating the functions of known cardiac transcription factors. Support for the latter explanation was provided by Morin and colleagues, who showed that MEF2 was able to strongly potentiate *Nppa* activation by GATA4, as discussed in Section IIIA of this chapter (Morin et al., 2000).

However, *Mef2c* mutant embryos still have normal expression of a number of other cardiac markers, including *Mlc2v* and *Mlc2a* (Lin et al., 1997). Since cardiac *Mlc2v* expression is MEF2-dependent (Navankasattusas et al., 1992), this finding suggested that other MEF2 proteins might compensate for the absence of MEF2C in the mutants. In support of this conclusion, there was a significant upregulation in the expression of *Mef2b* in *Mef2c* mutant embryos (Lin et al., 1997). The reason why other MEF2 isoforms can compensate for the lack of MEF2C on some cardiac promoters and not others is still not clear, but is strongly supported by observations that different MEF2 isoforms have distinct co-factor interactions.

The early lethality associated with cardiac defects in *Mef2c* mutants has necessitated more complex approaches to defining MEF2 requirements in later stages of vertebrate development. Recently, Schwarz and colleagues described a floxed conditional allele of *Mef2c* (Vong et al., 2005). General inactivation of this *Mef2c* conditional allele resulted in cardiac defects that were essentially identical to those described previously for the conventional *Mef2c* knockout (Lin et al., 1997; Vong et al., 2005). By contrast, later removal of *Mef2c* function in the heart using *Mlc2v*-Cre or *α-MHC*-Cre resulted in the development of viable offspring (Vong et al., 2005). Thus, it seems that the most critical requirement for MEF2C in the heart is early during cardiac development. It is possible that an initial wave of *Mef2c* expression is sufficient to initiate the expression of other *Mef2* genes, which may result in compensation and

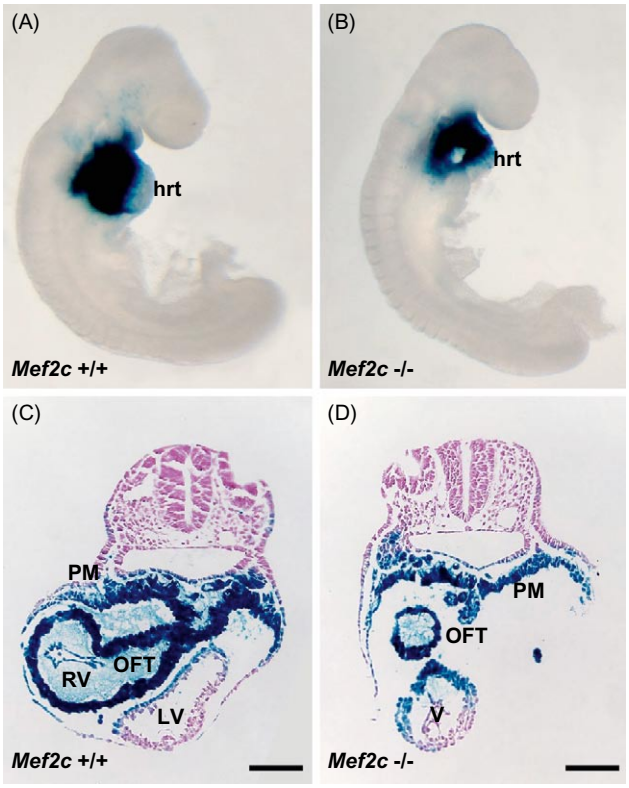

FIGURE 5 *Mef2c* is required for cardiac development in mice. Targeted inactivation of *Mef2c* in mice results in embryonic lethality at E10 due to severe cardiac morphogenetic defects. (A, B) E9.5 wild-type and knockout embryos are shown. Note the failed looping in the mutant animal shown in (B). (C, D) Transverse sections through wild-type and mutant animals showing the single ventricular chamber and a failure of looping morphogenesis in mutants. All embryos express β-galactosidase from the *Mef2c*-AHF-*lacZ* transgene, which shows the contribution of second heart field-derived cells to both wild-type and mutant hearts; second heart field contribution to the hearts of *Mef2c* mutant embryos is abnormal (compare staining in C and D) (hrt: heart; LV: left ventricle; OFT: outflow tract; PM: pharyngeal mesoderm; RV: right ventricle).

account for the lack of a cardiac phenotype in conditional knockout embryos with later *Mef2c* deletion. Alternatively, there may be an unappreciated role for *Mef2c* earlier in development or in another tissue, such as the vasculature, which then has a secondary effect on cardiac development in the global *Mef2c* knockout (Lin et al., 1998; Bi et al., 1999). Additional gene inactivation studies should resolve these issues in the future.

The *Mef2c* conditional allele was also utilized recently to define the function of *Mef2c* in the neural crest and its derivatives. Combination of the floxed *Mef2c* allele with *Wnt1*-Cre resulted in animals showing severe craniofacial abnormalities, ultimately causing neonatal mortality due to an occluded airway. Subsequent studies indicated that defective neural crest cell development arose due to a failure of *Mef2c* to activate the target genes *Dlx5*, *Dlx6* and *Hand2* in craniofacial mesenchyme (Verzi et al., 2007). Other recent studies using the zebrafish *hoover*

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(*hoo*) mutant, which has disrupted *Mef2c* function, also showed a requirement of *Mef2c* for craniofacial development upstream of the *Dlx* genes in an endothelin signaling-dependent pathway (Miller et al., 2007). Another role for *Mef2c*, in the formation of skeletal elements, was also identified using a conditional *Mef2c* allele in mice. Inactivation of *Mef2c* function in chondrocytes using a *Col2-Cre* transgene, or combined reduction of both *Mef2c* and *Mef2d* gene dosages, resulted in a failure of normal bone development (Arnold et al., 2007).

Targeted inactivation of *Mef2a* in mice also results in lethality due to cardiac defects, but homozygous *Mef2a* mutants survive embryogenesis and then the majority of mutants die shortly after birth (Naya et al., 2002). Affected animals showed severe right ventricular dilation and the activation of genes characteristic of cardiac hypertrophy and failure. *Mef2a*-null adult mice display significant dilated cardiomyopathy, without associated cardiac hypertrophy and have disorganized sarcomeres (Naya et al., 2002). Mutants that survived the neonatal period lived to adulthood, but showed defects in mitochondrial biogenesis, reflected by a three-fold reduction in mitochondrial gene copy number as assayed in the whole heart.

An analysis of gene expression comparing *Mef2a*-null to control mice identified several genes with aberrant expression, including *myospryn* and *myomaxin*, which each encode costameric proteins (Durham et al., 2006; Huang et al., 2006). These observations suggest the possibility that MEF2A coordinately regulates the expression of intermediate filament proteins, which are essential for cytoskeletal architecture. Disrupted costameric protein expression probably affects the stoichiometry of the components of the sarcomeres, and likely accounts for the disrupted cytoskeleton and cardiomyopathy in *Mef2a* knockout mice (Naya et al., 2002). Consistent with this idea, overexpression of either MEF2A or MEF2C in the adult myocardium results in dilated cardiomyopathy without significant hypertrophy (Xu et al., 2006). MEF2 overexpression in the heart resulted in significant sarcomeric disorganization, consistent with a role for MEF2 in costameric gene regulation and with the notion that alterations in costameric protein stoichiometry lead to dilated cardiomyopathy (Durham et al., 2006; Huang et al., 2006; Xu et al., 2006).

The observation that strikingly different phenotypes result from inactivation of *Mef2a* versus *Mef2c* or *Mef2d* underlines the distinct functions that each isoform must perform in the developing animal. A mouse knockout for *Mef2b* has yet to be described, but it will be interesting to determine if the phenotype of this mutant, once it is generated, is similar to the phenotypes of existing *Mef2* gene mutants. Furthermore, combination of individual knockouts into multiple knockout genotypes will provide more complete information on the roles of MEF2 factors in cardiac development. For example, given the strong upregulation of *Mef2b* transcription in *Mef2c* mutants (Lin et al., 1997; Vong et al., 2006), the phenotype of a $Mef2b^{-/-}$;$Mef2c^{-/-}$ double mutant should be most informative.

A number of other approaches have also been employed to define the role of MEF2 in muscle development. McDermott and colleagues generated a truncated form of MEF2A comprising the MADS and MEF2 domains, but lacking the majority of sequences C-terminal to those domains (Ornatsky et al., 1997). This molecule was predicted to bind to MEF2 target genes, but since it does not contain the transactivation domains, it is predicted to compete for activation of direct MEF2 target genes. Indeed, when this molecule was expressed in C2C12 skeletal myoblasts, there was an overall inhibition of muscle differentiation (Ornatsky et al., 1997). A similar approach was utilized by Karamboulas et al. who generated a MEF2C fusion with the transcriptional repressor domain of the Engrailed protein (EnR) from *Drosophila* (Karamboulas et al., 2006). When expressed in P19 embryonal carcinoma cells in a tissue culture cardiomyogenesis system, the chimeric MEF2 molecule inhibited the appearance of cardiomyocytes. When MEF2C-EnR was expressed in the developing hearts of transiently transgenic mouse embryos using the *Nkx2-5* promoter, some embryos showed defects in cardiac development (Karamboulas et al., 2006). The effects of this treatment ranged from an almost complete absence of differentiated myocardium to relatively normal embryos, presumably a reflection of different levels of MEF2C-EnR expression in the transiently transgenic embryos.

An advantage to the approaches utilizing dominant-negative or repressor versions of MEF2 are that such constructs probably interfere with the functions of all isoforms of MEF2 in the target cell, obviating the issues of genetic redundancy among MEF2 family members. Furthermore, utilization of tissue-specific promoters increases the specificity of the effects within the embryo. On the other hand, generation of MEF2-EnR chimeras is likely to show greater defects in cells than simply loss of MEF2 function. This is because MEF2 collaborates with a number of other factors on muscle-specific promoters. In addition, MEF2 often functions as a repressor, in which case MEF2-EnR may serve a gain-of-function role by hyper-repressing target genes.

IV.C. Direct Transcriptional Targets of MEF2 in the Heart

Since the initial identification of MEF2 factors as DNA-binding activities for muscle-specific promoters (Gossett et al., 1989), it is now apparent that MEF2 factors are present in a wide range of different cell types. As such, significant effort has been invested in defining direct transcriptional targets of MEF2. A current review of the literature indicates that there are over 80 known genes for

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TABLE 1 **A Subset of the Genes Identified as Direct MEF2 Targets in the Heart or Other Cardiovascular and Related Tissues (Columns Show the Name, Species, and Citations for the Indicated Genes)**

Gene	Species	References
Actin57B	*Drosophila*	Kelly et al., 2002
Aldolase A	Human	Hidaka et al., 1993; Salminen et al., 1995
Alpha cardiac actin	Rat, Mouse	Morin et al., 2000; Molinari et al., 2004;
		Lemonnier and Buckinham, 2004
Alpha cardiac MHC	Mouse	Molkentin and Markham, 1993, 1994; Adolph et al., 1993
Alpha MHC	Rat	Morin et al., 2000
Alpha T catenin	Human	Vanpoucke et al., 2004
Angiotensin II type Ia	Rat	Beason et al., 1999
Atrial natriuretic factor (Nppa)	Rat	Morin et al., 2000
BOP	Mouse	Phan et al., 2005
Brain natriuretic peptide (BNP)	Rat	Morin et al., 2000
CHAMP	Mouse	Liu et al., 2001
c-jun	Human, Rat	Han and Prywes, 1995; Nadruz et al., 2003
CPTI β	Rat	Baldan et al., 2004
Cytochrome c oxidase VIA	Mouse	Wan and Moreadith, 1995
Desmin	Mouse	Kuisk et al., 1996
Dlx5/6	Mouse	Verzi et al., 2007
Dystrophin	Human	Klamut et al., 1997
Gax	Mouse	Andres et al., 1995
GLUT4	Human	Thai et al., 1998; Mora and Pessin, 2000
HDAC9	Mouse	Haberland et al., 2007
HRC	Mouse	Anderson et al., 2004
Matrix Metalloproteinase 10 (MMP10)	Mouse	Chang et al., 2006
Mef2	*Drosophila*	Cripps et al., 2004
MicroRNA-1 (Mir1)	*Drosophila*	Sokol and Ambros, 2005
MLC2	*Xenopus*	Chambers et al., 1994
MLC2v (cardiac)	Chick, Rat	Goswami and Siddiqui, 1995; Navankasattusas et al., 1993
Muscle creatine kinase (MCK)	Mouse	Amacher et al., 1993; Cserjesi et al., 1994; Qin et al., 1997
Muscle LIM protein 60D	*Drosophila*	Stronach et al., 1999
Muscle LIM protein 84B	*Drosophila*	Stronach et al., 1999
Myocardin	Mouse	Creemers et al., 2006
Myomaxin	Mouse	Huang et al., 2006
Myospryn	Mouse	Durham et al., 2006
PGC1 alpha	Mouse	Czubryt et al., 2003; Handschin et al., 2003
Phosphoglycerate mutase	Human	Nakatsuji et al., 1992
Phosphoglycerate mutase	Rat	Ruizlozano et al., 1994; Nakatsuji et al., 1992
SERCA2	Rat	Moriscot et al., 1997
SRPK3	Mouse	Nakagawa et al., 2005
Troponin C (slow, cardiac)	Human, Mouse	Parmacek et al., 1994; Christensen and Kedes, 1999
Troponin I (cardiac)	Human, Rat	Di Lisi et al., 1998; Bhausar et al., 2000
Troponin T (cardiac)	Rat	Wang et al., 1994
Troponin I	*Drosophila*	Marin et al., 2004

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which promoter analyses have implicated MEF2 as a direct transcriptional activator. Table 1 contains a partial list of *bona fide* MEF2 target genes in the heart.

Recent genomic analyses have also begun to identify more globally the genes that are regulated by MEF2. Subtractive cloning assays were first used to define genes that were downregulated in *Mef2c* mutants, and were successful in identifying cardiac-restricted transcripts (Liu et al., 2001b). More recent microarray assays have been informative in delineating the physiological effects of MEF2 loss-of-function, and in defining potential MEF2 targets (Naya et al., 2002; Nakagawa et al., 2005; Sandmann et al., 2006). ChIP-on-Chip assays using anti-MEF2 antibodies have determined that there are as many as 670 nonoverlapping genomic regions bound by MEF2 in the developing *Drosophila* embryo (Sandmann et al., 2006, 2007). This large number of potential targets highlights the pervasive role that MEF2 plays in animal development.

In summary, the requirement for *Mef2* for muscle differentiation in *Drosophila* is well-established. In the absence of *Mef2* function, cardiac myocytes in the fly are properly specified, but fail to differentiate (Bour et al., 1995; Lilly et al., 1995). It is clear that MEF2 is also important for heart development and function in vertebrates, but the absolute requirement for myocyte differentiation is less clear. As discussed here, loss of *Mef2c* in mouse results in embryonic lethality due to severe morphogenetic defects in the heart, including a failure to undergo rightward looping (Lin et al., 1997). However, cardiac myocytes clearly form in the absence of *Mef2c*, and in general, myocytes are normally differentiated in those mutant mice (Lin et al., 1997, 1998; Bi et al., 1999; Vong et al., 2005, 2006). Similarly, loss of *Mef2a* function in mice causes perinatal lethality in the majority of mice due to cardiac defects, but again, myocytes differentiate properly in *Mef2a*-null animals (Naya et al., 2002). Zebrafish that lack the function of one or two *Mef2* genes have craniofacial and skeletal muscle defects, but no obvious cardiac defects, and muscle differentiation in the cardiac and skeletal muscle lineages appear normal (Hinits and Hughes, 2007; Miller et al., 2007).

Given the many commonalities in MEF2-dependent transcriptional pathways that have been conserved between flies and vertebrates, it is reasonable to expect that MEF2 is also required for differentiation of myocytes in vertebrates. A likely explanation for the less severe differentiation phenotypes in mouse and fish *Mef2* gene knockouts/knockdowns is the partial redundancy in *Mef2* gene function due to the presence of four *Mef2* genes in vertebrates compared to invertebrates. Thus, the question remains as to whether MEF2 is really required for myocyte differentiation in vertebrates. As noted earlier in this chapter, studies using dominant-negative forms of MEF2 in C2C12 skeletal myoblasts in culture have strongly implicated a requirement for MEF2 in myoblast differentiation in that system (Ornatsky et al., 1997), and studies using a repressor form of MEF2 in P19 cells also suggest an important role for MEF2 in cardiac muscle differentiation (Karamboulas et al., 2006). Ultimate genetic testing of the requirement of MEF2 for cardiomyocyte differentiation in vertebrates will require additional compound mouse knockouts. It will be important to use multiple approaches to understand the function of *Mef2* genes *in vivo*, including gene disruption, dominant-negative and siRNA approaches. This combined strategy should ultimately elucidate the complex function of MEF2 in the many tissues where *Mef2* genes are expressed in vertebrates.

V. REGULATION OF *MEF2* GENE TRANSCRIPTION

V.A. *Mef2* Gene Regulation as a Paradigm for Modular Transcriptional Control

Mef2 transcripts show remarkably broad expression in muscle lineages during embryogenesis. While we are now aware that MEF2 expression and function are

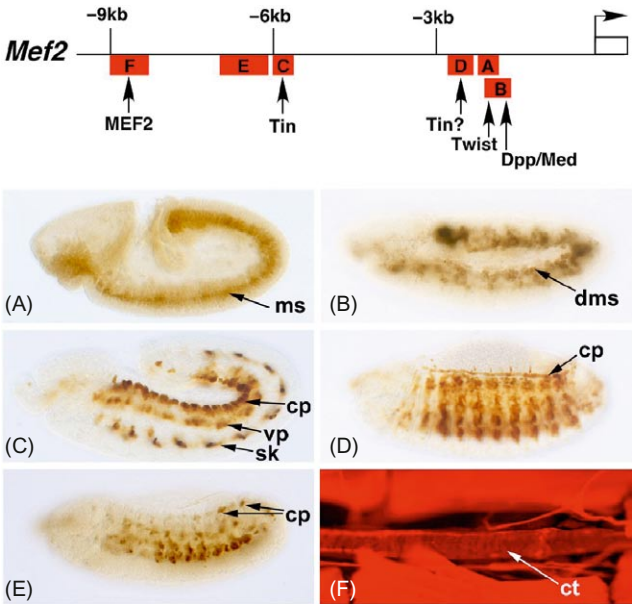

FIGURE 6 *Cis*-regulatory elements of the *Drosophila Mef2* gene. Top: Schematic of the upstream region of *Mef2* in *Drosophila*. The locations of specific enhancers discussed in the text are shown, as well as the transcription factors that regulate these enhancers. (A–F) Activity of *Mef2* enhancers visualized by staining for β-galactosidase accumulation in transgenic embryos containing *Mef2-lacZ* fusions. (A) At stage 9 the earliest enhancer is active throughout the invaginating mesoderm. (B) At stage 10 a dorsal mesoderm enhancer, responsive to Dpp, is active. (C) At stage 12, the Tin-dependent enhancer is active in precursors of cardiac muscle (ca), visceral muscle (vm) and a subset of skeletal muscles (sk). (D) At stage 14, a late cardiac enhancer is active, predominantly in Tin cells of the dorsal vessel. (E) An enhancer active in the *svp*-expression cells is active at stage 14. (F) The *Mef2* autoregulatory enhancer functions during larval development in the cardiac tube. All images show anterior to the left and dorsal side uppermost.

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extensively regulated post-transcriptionally, early RNA *in situ* hybridization studies indicated that a major component of *Mef2* gene regulation occurs at the level of transcription. As has been observed for other regulatory genes that have essential and broad functions during embryonic development, *Mef2 cis*-regulatory regions are composed of a large number of dispersed and independently-functioning enhancers. In *Drosophila*, the *Mef2* transcribed region spans 15 kb, and most of the *Drosophila Mef2* enhancers that have been described are contained within a region of ~16 kb upstream of the gene (Fig. 6) (Schulz et al., 1996; Nguyen and Xu, 1998). Murine *Mef2c* regulatory elements appear to be encompassed by a region spanning approximately 200 kb, which also encompasses the transcribed portion of the gene (Fig. 7) (Dodou et al., 2004).

Thus, the dynamic pattern of *Mef2* gene expression observed in animals arises from the combined actions of a number of independently-functioning regulatory modules that together provide the spatial and temporal signals for *Mef2* transcription. Given the fact that *Mef2* expression is strongly detected during the specification of multiple lineages, it is likely that that transcription factors that regulate each distinct *Mef2* enhancer are themselves critical regulators of cell fate. For this reason, significant effort has focused on the identification and characterization of *Mef2* enhancer sequences.

V.B. Regulation of *Mef2* Transcription in the *Drosophila* Heart

In *Drosophila* embryos, cells contributing to the mature cardiac tube arise as a result of a series of tightly-regulated transcriptional, cell signaling and morphological events (Cripps and Olson, 2002). Presumptive mesodermal cells are specified on the ventral surface of the blastoderm embryo by the influence of the maternal gene product Dorsal, and the activation of the mesodermal determinant Twist. The mesodermal cells subsequently invaginate and spread laterally and dorsally along the ectoderm. Mesodermal cells on each side of the embryo are defined as dorsal mesoderm when they are in proximity with the dorsal ectoderm and receive the TGFβ molecule Decapentaplegic (Dpp) (Frasch, 1995).

Within the dorsal mesoderm, expression of the NK homeodomain transcription factor Tinman (Tin) is responsible for the specification of cardiac mesoderm. Subsequently, a number of inductive processes subdivide the cardiac mesoderm into specific cell types (Frasch, 1995, 1999). A major decision at this point is the designation of cardial cells that either continue to express *tin*, or activate expression of the orphan nuclear receptor gene *seven-up* (*svp*) and downregulate *tin* transcription (Gajewski et al., 2000; Lo and Frasch, 2001; Lovato et al., 2002). Following these important cell fate decisions, the

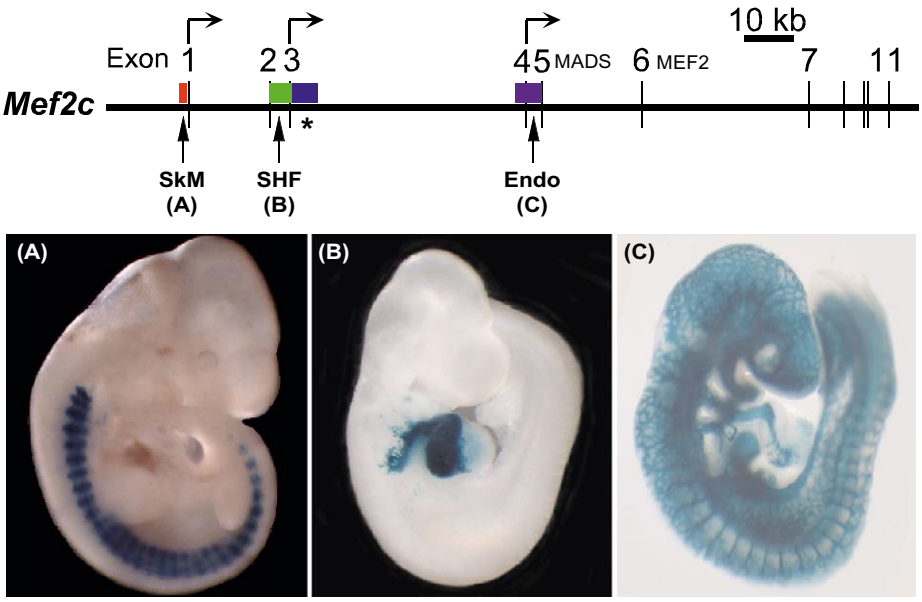

FIGURE 7 *Cis*-regulatory elements of the mouse *Mef2c* gene. The top shows a schematic of the upstream region of the mouse *Mef2c* gene. The locations of specific enhancers discussed in the text are shown as colored rectangles (Red: skeletal muscle (SkM); Green: Isl1-dependent SHF enhancer; Blue: Nkx2-5/FoxH1-dependent SHF enhancer; Purple: endothelial (Endo) enhancer). Photographs of X-gal stained transgenic embryos with the SkM, SHF and Endo enhancers directing the expression of β-galactosidase are shown in (A), (B) and (C), respectively. An E11.5 embryo is shown in (A); E9.5 embryos are shown in (B) and (C). Known transcriptional starry sites are depicted on the schematic as arrows; exons are depicted as vertical black lines.

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two rows of cardial cells on either side of the embryo migrate dorsally and meet at the midline to form a linear cardiac tube (Cripps and Olson, 2002).

Studies over the past 10 years have identified *Mef2* enhancers with activities that overlap temporally and spatially with each of the important morphological and regulatory events contributing to cardiac tube formation, and their activities are shown in Fig. 6. The earliest-acting *Mef2* enhancer is responsible for uniform expression throughout the newly-specified mesoderm (Lilly et al., 1995). Genetic studies indicate that the earliest expression of *Mef2* might be dependent on the mesodermal determinant Twist, since both gain and loss of Twist activity results in concomitant alterations in the pattern of early *Mef2* expression (Lilly et al., 1994; Nguyen et al., 1994; Taylor, 1995). The early expression of *Mef2* in the mesoderm is controlled by a proximal regulatory region approximately 2 kb upstream of the transcriptional start site (Fig. 6A) (Lilly et al., 1995; Nguyen and Xu, 1998). Sequences within this region show strong binding to Twist protein *in vitro*, and ectopic Twist activates the enhancer (Cripps et al., 1998; Nguyen and Xu, 1998).

Adjacent to the Twist-responsive enhancer, and possibly overlapping with it, is a *Mef2* regulatory region that directs expression to the dorsal mesoderm (Fig. 6B). Dorsal mesoderm is specified in the *Drosophila* embryo by ectodermal Dpp signaling (Frasch, 1995, 1999). Not surprisingly, the *Mef2* dorsal mesoderm enhancer is highly responsive both to Dpp levels and to levels of the *Drosophila* Smads orthologs, Medea and Mad (Nguyen and Xu, 1998). Medea and Mad bind robustly to sequences within the *Mef2* dorsal mesoderm enhancer, but it is not known if the Medea binding sequences are required for enhancer activity (Nguyen and Xu, 1998). The *Mef2* dorsal mesoderm enhancer also contains the Twist sites required for early mesoderm expression of *Mef2*, although it is not clear if these Twist sites contribute to the function of this enhancer (Nguyen and Xu, 1998).

As cells within the dorsal mesoderm become specified to a cardiac fate, a third *Mef2* enhancer is active in the precardiac cells, beginning around stage 11 (Fig. 6C). This enhancer is located further upstream, at approximately −5.8 kb, and can direct reporter gene activity in precardiac cells of the fly embryo, precursors of the trunk visceral mesoderm and a subset of skeletal muscles (Gajewski et al., 1997, 1998; Cripps et al., 1999). The enhancer contains two binding sites for the NK homeodomain factor Tin, and mutation of either or both Tin sites completely ablates the activity of this enhancer in all three of the muscle lineages (Gajewski et al., 1997, 1998; Cripps et al., 1999).

At stage 14, another *Mef2* enhancer predominantly active in the Tin cardiac cells, has been identified (Fig. 6D) (Nguyen and Xu, 1998). This enhancer appears to support strong reporter gene activity in the cardiac tissue through the end of embryogenesis. Factors regulating the activity of this enhancer have yet to be defined, although the region does contain a consensus-binding site for the Tin protein.

From stage 13 onwards, the maturing dorsal vessel comprises mutually exclusive *tin* and *svp*-expressing cardial cells (Gajewski et al., 2000; Ward and Skeath, 2000; Lo and Frasch, 2003). Therefore, it was reasonable to postulate that a *Mef2* enhancer active in the *svp*-expressing cells of the dorsal vessel must exist. Early data suggested that this was a relatively distal enhancer (Schulz et al., 1996), and sequences responsible for this activity have now been identified (Fig. 6E) (Gajewski et al., 2000). Deletional analyses indicate that the regulation of this region is complex, since several discrete elements are required for enhancer activity, and transcriptional regulators of this sequence have yet to be identified (Gajewski et al., 2000).

A final *Mef2* enhancer that is active in cardiac tissue in *Drosophila* shows a relatively broad pattern of activity in the mature muscles of the developing animal. This late muscle enhancer is located at approximately −9 kb relative to the transcriptional start site, and becomes active in visceral and skeletal muscle during embryogenesis (Nguyen and Xu, 1998: Cripps et al., 2004). Cardiac activity of this enhancer is not detected until larval life (Fig. 6F). The function of this late heart enhancer depends specifically on the integrity of a binding site for MEF2 itself, indicating that the *Mef2* gene is subject to positive autoregulation (Cripps et al., 2004). This enhancer probably functions as a late-maintenance element for mature muscles.

The activities of the six *Drosophila Mef2* enhancers described above provide a detailed and discrete description of the fate of cardiac cells, from the time when the cells are first specified as mesoderm in the embryo to the time when they power circulation in the developing larva. As indicated above, regulators of *Mef2* transcription are critical regulators of mesodermal cell fate decisions, underlining the importance of MEF2 in regulating mesodermal development. For most of these *Drosophila* enhancers, only single regulators have been identified to date. This suggests that additional critical muscle-specific activators of *Mef2* are still to be identified, since the majority of well-characterized enhancers are activated combinatorially. Future studies should resolve how the activities of these many distinct enhancers are integrated to give the complex and precise pattern of *Mef2* expression in *Drosophila*.

V.C. Regulation of Mef2c Transcription in the Mammalian Heart

The large and complex organization of mammalian *Mef2* genes has hindered the identification of regulators of *Mef2* transcription in higher animals, although recent studies are providing critical insight into the regulatory elements of the mouse *Mef2c* gene. Mouse *Mef2c* spans almost 200 kb,

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and comprises at least 14 exons. Alternative promoters are used to generate transcripts that are restricted to skeletal muscle, heart or brain (Fig. 7). Each of these alternate first exons are spliced to common exons encoding the MADS and MEF2 domains (Wang et al., 2001; Zhu and Gulick, 2004; Zhu et al., 2005).

The first *Mef2c* enhancer that was described was identified based on its proximity to a skeletal muscle-specific exon (Wang et al., 2001). Accordingly, this enhancer is active in all skeletal muscles and their precursors, beginning at E9.0 (Fig. 7A). Deletion and mutagenesis studies of this sequence revealed a critical requirement for a MyoD E box (Wang et al., 2001; Dodou et al., 2003). In addition, an AT-rich sequence, which might comprise a MEF2 binding site, was also required for maximal activation of the enhancer (Wang et al., 2001; Dodou et al., 2003). While this enhancer is not activated in cardiac tissue, these studies importantly demonstrated that mammalian *Mef2* genes are likely to be regulated by dispersed modular enhancers, similar to the situation observed for *Drosophila*.

Mef2c-regulatory regions that show activity in cardiovascular tissues have more recently been identified. One element, located adjacent to the heart-specific exon identified by Wang et al. is responsible for activation of *Mef2c* transcription in the anterior, or second, heart field (Fig. 7B) (Wang et al., 2001; Dodou et al., 2004) (see Chapter 2.2). This enhancer, termed *Mef2c* AHF, becomes activated very early during cardiac cell commitment at E7.5, and its activity is restricted to cells of the second heart field and its descendants (Dodou et al., 2004). The function of this enhancer is absolutely dependent on the integrity of binding sites for GATA factors and the LIM-homeodomain transcription factor Islet-1 (Dodou et al., 2004), which has been shown recently to function at or near the top of a transcriptional network for second heart field development (Cai et al., 2003; Black, 2007).

The isolation and characterization of the *Mef2c* AHF enhancer has enabled detailed studies of the second heart field and its contributions to the heart. By combining transgenic mice in which the *Mef2c* enhancer is fused to Cre-recombinase, with mice carrying floxed alleles of a variety of genes, studies ranging from lineage tracing analyses (Verzi et al., 2005) to tissue-specific knockouts (Ai et al., 2006, 2007; Park et al., 2006; Goddeeris et al., 2007) have been achieved. Identification of other *Mef2* enhancers showing specificity to other important cell lineages will likely provide similar essential reagents for studying cardiac development.

A third *Mef2c* enhancer, located just upstream of exon 4, activates reporter gene expression broadly in the vascular endothelial cells of transgenic animals, but not in vascular smooth muscle cells (De Val et al., 2004). *In vivo*, the enhancer first becomes active in blood islands of the yolk sac at E7.5. Subsequently, the *Mef2c* F7 endothelial enhancer becomes active throughout the vascular endothelium during embryogenesis, and in most adult vascular endothelial cells (Fig. 7C). Mutational analyses of the enhancer revealed a requirement for Ets transcription factor binding sites for enhancer function *in vivo* (De Val et al., 2004). Given the broad requirement for Ets proteins in the formation of vascular endothelial cells (Parmacek, 2001), and given the requirement for *Mef2c* function in vascular development (Lin et al., 1998; Bi et al., 1999), the identification of *Mef2c* as a target of Ets factors defines an important component of the gene regulatory network for the specification and differentiation of vascular endothelial cells.

More recently, an additional endothelial-specific enhancer from the mouse *Mef2c* gene was identified (De Val et al., 2008). This enhancer, termed *Mef2c* F10, is activated exclusively in endothelial cells and their progenitors, beginning at the blood island stage (E7.5), but initiates prior to *Mef2c* F7 (De Val et al., 2004, 2008). *Mef2c* F10 contains a deeply conserved 44 bp core element that is both necessary and sufficient for enhancer function *in vivo*. This small region contains a composite-binding motif for Forkhead and Ets transcription factors, and this motif is simultaneously bound and synergistically activated by the Forkhead protein FoxC2 and the Ets protein Etv2 (De Val et al., 2008). Interestingly, the FOX:ETS motif, first discovered in *Mef2c*, is prevalent throughout the human genome, and is strongly-associated with endothelial genes and enhancers. All FOX:ETS-containing enhancers tested to date are synergistically activated by FoxC2 and Etv2, and these two transcription factors are sufficient to induce ectopic vasculogenesis in *Xenopus* embryos (De Val et al., 2008), suggesting that combinatorial activation of *Mef2c* by FoxC2 and Etv2 is part of a much broader cooperative mechanism for endothelial gene activation.

By investigating the role of *Foxh1* in the formation of the second heart field, von Both et al. showed that *Foxh1*$^{-/-}$ embryos displayed cardiac defects highly similar to those observed for *Mef2c* mutants, including failure to form the right ventricle and outflow tract (von Both et al., 2004). To determine if this result arose from a direct transcriptional interaction between FoxH1 and the *Mef2c* gene, the authors searched for candidate FoxH1-binding regions in *Mef2c*. These analyses identified an additional putative enhancer responsive to FoxH1 and Nkx2-5 (Fig. 7) (von Both et al., 2004). These observations are potentially interesting, since mutations that affect two different genes show similar phenotypes, and this similarity may be due to a direct transcriptional regulatory interaction between the two genes. Second, the identification of two distinct second heart field enhancers from the *Mef2c* gene raises the possibility that there might be redundant enhancers functioning for this cell type. Alternatively, the expression patterns of the two enhancers might, on further investigation, show subtle yet important functional differences. Third, the activation of *Mef2c* by Nkx2-5 is highly reminiscent of the activation of *Drosophila*

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Mef2 in cardiac precursors by the *Drosophila* NK homeodomain factor Tinman, underlining the ancient evolution of the transcriptional network that functions during cardiac development (see Chapter 1.2). It will be interesting to identify additional cardiac enhancers from *Mef2c* in order to determine how all of these elements are coordinated and integrated to govern *Mef2c* expression in the heart.

The *Mef2* gene transcriptional regulatory modules described in this chapter have been studied in isolation, following fusion of a wild-type or mutant enhancer to a reporter gene and analysis of reporter gene expression in transgenic animals. Whether there is any interplay among discrete enhancers that show partially-overlapping temporal or spatial activities, or whether the activation of one enhancer sequence specifically affects the activation of another has yet to be determined. Furthermore, the nature of global chromatin organization at the *Mef2* gene loci has yet to be defined in detail.

Why should *Mef2* genes be subject to such complex transcriptional regulation? Given the broad expression of *Mef2*, it might seem more efficient if *Mef2* transcription were activated and then subjected to positive autoregulation to maintain its expression. However, this mechanism might be too rigid, since there are a number of instances where the expression of specific *Mef2* genes is lost from a particular tissue or lineage. For example, all cells of the *Drosophila* dorsal vessel arise from the MEF2-positive dorsal mesoderm; however, the accessory pericardial cells downregulate *Mef2* expression and are MEF2-negative in mature embryos (Cripps and Olson, 2002). Therefore, the complexity of *Mef2* gene regulation probably allows for greater modulation of levels of gene transcription as new cell types are specified and subsequently differentiate. How the regulation of the many distinct enhancers from all of the various *Mef2* genes are integrated to give rise to the endogenous pattern of *Mef2* transcription are important questions that remain to be resolved by future studies.

VI. FUTURE DIRECTIONS

MEF2 proteins function as a transcriptional switch in many diverse lineages during development and in adulthood. To accomplish this, MEF2 has evolved numerous regulatory interactions. As discussed in this chapter, MEF2 interacts with a wide array of co-factors, including both positive and negative regulators of transcription. MEF2 serves as an essential binding platform for enzymes that influence chromatin structure, including histone acetylases and deacetylases. Similarly, MEF2 serves as a platform for potent transcriptional regulators that do not directly contact DNA themselves, such as myocardin and MASTR. How does MEF2 interact with such a diverse group of co-factors and accomplish seemingly opposing influences on transcription, in some cases acting as a repressor and in others as an activator? In part, the answer to this question appears to lie in the post-translational regulation of MEF2 by many diverse signaling pathways. Numerous different signaling pathways that influence MEF2 function and protein–protein interactions via phosphorylation were discussed in this chapter. However, many of these pathways function in the same cell types and in response to the same signals. Thus, it still remains unclear how MEF2 integrates and interprets these distinct and often opposing inputs to control transcription into precise developmental or physiological outputs. Future investigation in this area will certainly focus on defining how various post-translational modifications of MEF2 interact with and influence each other. In turn, it will be important to determine how post-translational modifications of MEF2 affect its structure and protein–protein interactions.

Given the fundamental role of MEF2 proteins in so many biological processes, it is not surprising that MEF2 expression and function is tightly-regulated, and that this regulation is complex and occurs at many levels. Much has been learned over the last decade about the post-translational regulation of MEF2 function, and we are also beginning to decipher the complex transcriptional regulation of *Mef2* genes. However, much remains to be defined about the regulation of this transcription factor family. As noted above, it is still not clear how the many post-translational modifications of MEF2 are integrated. It is also not clear how the many modular enhancers of *Mef2c* work in the context of the genome, and nothing is known currently about the transcriptional regulation of other vertebrate *Mef2* genes. Recent studies have uncovered a fundamental regulatory role for microRNAs in muscle gene regulation by targeting 3′ untranslated regions (UTRs) of muscle genes (Zhao et al., 2005b; Chen et al., 2006; van Rooij et al., 2006; Callis et al., 2007; van Rooij and Olson, 2007; van Rooij et al., 2007). Interestingly, the 3′UTRs of the vertebrate *Mef2* genes are evolutionarily conserved, suggesting a possible biological function. Indeed, the 3′UTR of the *Mef2a* transcript has been shown to be a *cis*-acting translational repressor, although the mechanism is not clear (Black et al., 1997). It remains to be determined whether the *Mef2a* 3′UTR or the UTRs of other *Mef2* genes are regulated by microRNAs, although it seems likely since fine-tuned control of *Mef2* gene expression and function appears to occur at almost every other level of regulation.

Mutations that result in haploinsufficiency of any of several transcription factor genes are known to cause or contribute to human disease. These include genes for several transcription factors that function in the core cardiac network, including *TBX5*, *GATA4* and *NKX2-5* (Basson et al., 1997; Schott et al., 1998; Pehlivan et al., 1999; Giglio et al., 2000; Garg et al., 2003; Mori and Bruneau, 2004; Schluterman et al., 2007; Pabst et al., 2008). Interestingly, MEF2 family members also function in the core network, and as noted in this chapter, MEF2C has been shown to

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interact transcriptionally or physically with Nkx2-5, GATA4 and HAND2 (Skerjanc et al., 1998; Morin et al., 2000; Vanpoucke et al., 2004; Zang et al., 2004). Together, these observations suggest that mutations in human *MEF2* genes may also influence heart development or function. Furthermore, the role of MEF2 transcription factors in hypertrophic responsiveness and cardiomyopathy in response to pathologic stimuli in mice is well-established (Backs and Olson, 2006; Liu and Olson, 2006; Olson et al., 2006). Thus, it is reasonable to expect that *MEF2* genes may be involved in human heart failure as well. Indeed, some studies have suggested a role for *MEF2A* in inherited coronary artery disease in humans where a 21 bp deletion in the *MEF2A* gene may be correlated with late-onset disease (Wang et al., 2003a; Bhagavatula et al., 2004). However, other studies have suggested that the 21 bp deletion, which results in an in-frame loss of 7 amino acids in the C-terminus of MEF2A, is a rare but normal allelic variant (Altshuler and Hirschhorn, 2005; Kajimoto et al., 2005; Wang et al., 2005; Weng et al., 2005; Horan et al., 2006). Therefore, it still remains unclear what role, if any, *MEF2* genes may play in human cardiovascular disease. This will be an important area of future investigation, and human genetic studies may identify even more previously unappreciated roles for MEF2 transcription factors in development and disease.

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