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Cabin1 Represses MEF2-Dependent Nur77 Expression and T Cell Apoptosis by Controlling Association of Histone Deacetylases and Acetylases with MEF2

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Summary

TCR signaling leading to thymocyte apoptosis is mediated through the expression of the Nur77 family of orphan nuclear receptors. MEF2 has been shown to be the major transcription factor responsible for calcium-dependent Nur77 transcription. Cabin1 was recently identified as a transcriptional repressor of MEF2, which can be released from MEF2 in a calcium-dependent fashion. The molecular basis of repression of MEF2 by Cabin1, however, has remained unknown. We report that Cabin1 represses MEF2 by two distinct mechanisms. Cabin1 recruits mSin3 and its associated histone deacetylases 1 and 2; Cabin1 also competes with p300 for binding to MEF2. Thus, activation of MEF2 and the consequent transcription of Nur77 are controlled by the association of MEF2 with the histone deacetylases via the calcium-dependent repressor Cabin1.

Introduction

TCR-mediated thymocyte negative selection in the form of apoptosis is important for elimination of self-reactive T cells (Winoto, 1997; Wong and Choi, 1997; Sebzda et al., 1999). Among the proteins that are induced by TCR signaling is the Nur77 family of nuclear receptors, which have been shown to be crucial for TCR-mediated thymocyte apoptosis (Liu et al., 1994a; Woronicz et al., 1994). In an effort to dissect the signal transduction pathway leading from TCR to Nur77 transcription, Winoto and colleagues identified two calcium-responsive DNA elements in the Nur77 promoter that were consensus binding sites for the transcription factor MEF2 (Woronicz et al., 1994, 1995). This suggested that MEF2 might play a key role in mediating intracellular calcium signaling to activate Nur77 transcription.

The MEF2 family of transcription factors is ubiquitously expressed in metazoans and plays pivotal roles in diverse physiological processes (Black and Olsen, 1998). MEF2 was first identified in muscle cells essential for muscle differentiation during development (Gossett et al., 1989; Pollock and Treisman, 1991; Yu et al., 1992; Leifer et al., 1993; Martin et al., 1993, 1994; McDermott et al., 1993). Recently, more and more nonmuscle functions have been uncovered for MEF2, including T cell receptor–mediated apoptosis of thymocytes (Woronicz et al., 1994, 1995), maintenance of postmitotic neuronal survival (Mao et al., 1999), mitogenic response to serum (Han and Prywes, 1995), monocytic response to bacterial lipopolysaccharides (Han et al., 1997), and glucose metabolism (Liu et al., 1994b). Although all four MEF2 isoforms, MEF2A–D, are expressed in many nonmuscle cells, including thymocytes, they lack transcriptional activity, implicating the existence of negative regulators for MEF2 in these cell types (Ornatsky and McDermott, 1996; Black and Olsen, 1998). A multitude of intracellular signaling pathways has been shown to activate MEF2, including increases in intracellular calcium concentration (Black and Olsen, 1998). Taken together, these observations suggest that MEF2 may undergo a calcium-dependent switch from a transcriptionally repressed state to an activated state.

Positive and negative regulation of eukaryotic transcription has been shown to be mediated in part by two opposing types of enzymatic activities, histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Grunstein, 1997; Hassig and Schreiber, 1997; Grant et al., 1998; Kuo and Allis, 1998). While HATs are associated with activation of gene transcription, HDACs mediate transcriptional repression. The switch of a promoter from an "off" to an "on" state is often caused by dissociation of HDACs from, and the subsequent association of HATs with, specific DNA binding transcription factors. Several distinct modes of recruitment of HATs and HDACs to chromatin-bound transcription factors have been reported. For example, the transcription factor c-Myc is kept inactive by Mad, which recruits the corepressor mSin3 and the associated HDACs. Additionally, Mad competes for the c-Myc binding sites on the promoter and for the binding of the partner protein Max (Ayer et al., 1995; Laherty et al., 1997). Unlike the c-Myc family of transcriptional regulators, the nuclear hormone receptors are associated with HATs and HDACs in a ligand-dependent fashion. Thus, unbound hormone receptors are associated with HDACs via the corepressor N-CoR and SMRT (Alland et al., 1997; Heinzel et al., 1997; Nagy et al., 1997). Binding of hormones to the receptor leads to a conformational change in the receptor, causing the dissociation of the corepressor complex and the association of the activator complex, including p300/CBP (Janknecht and Hunter, 1996). Unlike c-Myc and nuclear hormone receptors, the calcium-dependent activation of MEF2 seems to operate through a distinct mechanism.

We recently identified a corepressor of the MEF2 family of transcription factors known as Cabin1 (Sun et al., 1998), or Cain in rat (Lai et al., 1998) (refer hereafter as Cabin1). Association of Cabin1 with MEF2 represses its transcriptional activity. This repression is relieved by activated calmodulin, which binds to Cabin1, releasing it from MEF2 (Youn et al., 1999). The calcium-dependent regulation of MEF2 by Cabin1 provides one mechanism of regulation of a transcription factor by calcium. However, how Cabin1 suppresses MEF2's transcriptional activity has remained unknown. We report herein that Cabin1 represses MEF2 transcriptional activity by at least two distinct mechanisms. First, Cabin1 recruits

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mSin3 along with its associated HDAC1 and HDAC2. The association of HDACs with Cabin1 accounts for most of its repressive activity toward MEF2, as this repression can be reversed by the histone deacetylase inhibitor trichostatin A. Second, binding of Cabin1 to MEF2 at the N-terminal MADS/MEF2 domain competes against the coactivator p300 for MEF2 binding in the absence of calcium. An increase in $[Ca^{2+}]_i$ leads to the dissociation of Cabin1 from MEF2, as shown previously (Youn et al., 1999), and association of p300, which mediates transcriptional activation of MEF2 target genes.

Results

MEF2 Is Constitutively Associated with Its Cognate DNA Binding Sites in the Nur77 Promoter

It has been shown that MEF2 proteins expressed in nonmuscle cells are transcriptionally inactive, even though they are localized in the nucleus and are fully competent in DNA binding (Ornatsky and McDermott, 1996). In particular, it has been demonstrated that MEF2 DNA binding activity is present in unstimulated T cells, and this activity does not change upon T cell activation (Woronicz et al., 1995). One possible explanation for these observations is that the nuclearly localized MEF2 is sequestered away from its target promoters. An alternative explanation is that MEF2 is bound to DNA but is kept transcriptionally inactive by chromatin-modifying repressors. To distinguish between these two possibilities, we performed the chromatin immunoprecipitation-PCR (CHIP) assay. We expressed in Jurkat T cells MEF2D and Cabin1 together with pGLNur77(−307 to −242), a reporter plasmid that contains two MEF2 binding sites within the minimal Nur77 promoter. Upon chemical cross-linking, MEF2D or Cabin1 was immunoprecipitated from cell lysates and the bound pGLNur77 (−307 to −242) was detected by PCR amplification of the MEF2-containing fragment spanning nucleotides −307 to −204 (Figure 1A). MEF2D bound to the MEF2 binding sites in the Nur77 promoter independent of intracellular calcium concentration (Figure 1B). Similarly, Cabin1 was found to be associated with the MEF2-containing Nur77 minimal promoter, presumably via MEF2, in the presence of EGTA. This association is abrogated by the addition of Ca^{2+}, consistent with the calcium-sensitive dissociation of Cabin1 from MEF2. These results are consistent with the possibility that MEF2 is constitutively bound to DNA, and its transcriptional silence in the absence of stimuli strongly suggests the existence of a repressor such as Cabin1.

Cabin1 Recruits Histone Deacetylases 1 and 2 via mSin3a

Histone deacetylases and the corepressor mSin3 have been shown to confer inhibition of transcription through their association with DNA binding transcription factors (for a recent review, see Grunstein, 1997). As Cabin1 inhibits the transcriptional activity of MEF2, we investigated whether Cabin1 is associated with HDACs. Thus, HDAC1 and HDAC2 tagged with a Flag epitope at their N termini were coexpressed with a c-myc epitope–tagged

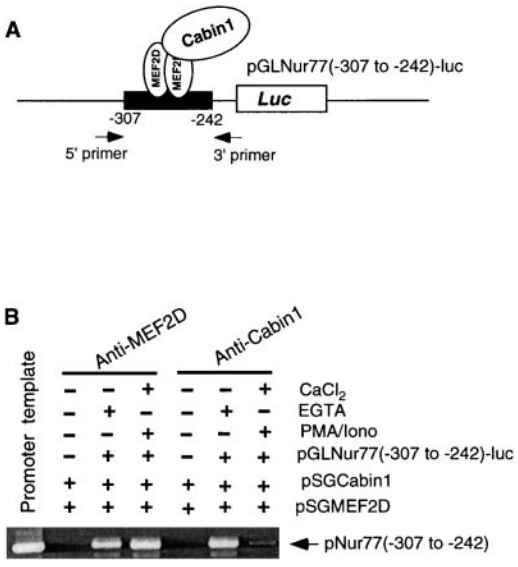

Figure 1. Dependence on Ca^{2+} of MEF2 and Cabin1 Binding to the Nur77 Promoter

(A) Schematic diagram of the CHIP assay.

(B) Jurkat T cells were transfected with pSGMEF2D, pSGCabin1, with or without pGLNur77(−307 to −242)-luc. Nuclear lysates were incubated with either anti-MEF2D or anti-Cabin1 antibodies. Immunoprecipitated DNA was amplified by PCR using primers specific to the promoter region of Nur77 encoded by the plasmid.

Cabin1 in Jurkat T cells. Cabin1 was found to coimmunoprecipitate with both HDAC1 and HDAC2 (Figure 2A, lanes 2 and 4). As both HDAC1 and HDAC2 are known to bind to the corepressor mSin3, we then determined whether Cabin1 binds to HDAC1 and HDAC2 directly or indirectly through mSin3. Thus, ^{35}S-labeled mSin3 synthesized by an in vitro transcription-translation system was mixed with Jurkat T cell extract containing c-myc-Cabin1. A signal for mSin3A was detected in the immunoprecipitate of Cabin1 (Figure 2B), suggesting that HDAC1 and HDAC2 is bound to Cabin1 via mSin3. In addition, we have also been able to coimmunoprecipitate endogenous Cabin1 and endogenous HDAC1 or HDAC2 but not endogenous HDAC4 (Figure 2C), providing further support for the association between HDAC1 or HDAC2 and Cabin1 in vivo. Together, these results suggest that Cabin1 recruits HDAC1 and HDAC2 through direct binding of mSin3 in a manner that is similar to several other known transcriptional corepressors (Alland et al., 1997; Hassig et al., 1997; Heinzel et al., 1997; Kadosh and Struhl, 1997; Laherty et al., 1997; Nagy et al., 1997; Zhang et al., 1997).

The mSin3A binding domain in Cabin1 was mapped by coimmunoprecipitation between mSin3A and various Cabin1 deletion mutants. Both full-length Cabin1 and Cabin1(1–1800) coimmunoprecipitated with mSin3A (Figures 3A and 3B). Deletion of the N-terminal 319 amino acids [Cabin1(320–2220)], however, abrogated the binding, suggesting that this N-terminal domain is necessary for Cabin1-mSin3A interaction. When Cabin1(1–315) was coexpressed with mSin3A, the two proteins coimmunoprecipitated, indicating that the N-terminal 315

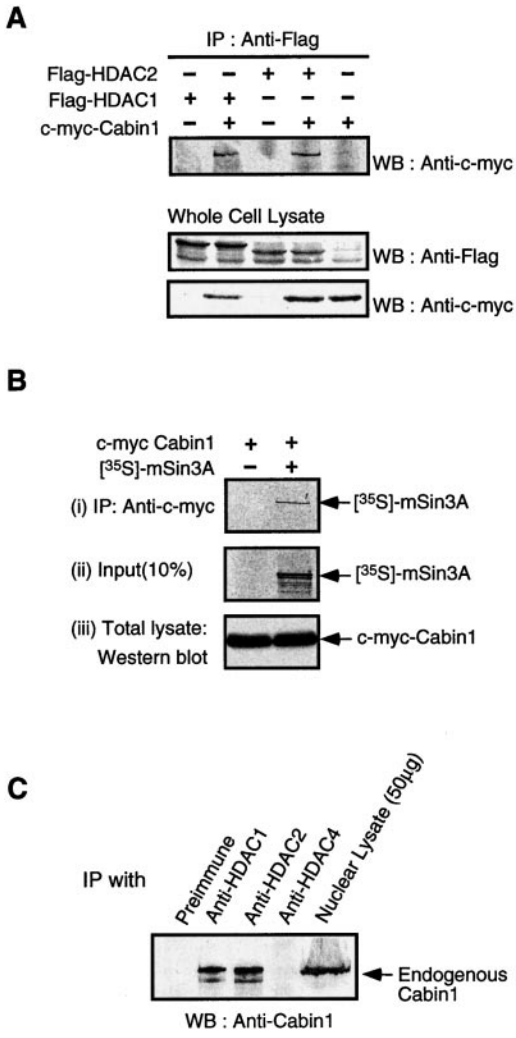

Figure 2. Cabin1 Interacts with Histone Deacetylases and mSin3A

(A) Cabin1 binds to HDAC1 and 2. Jurkat T cells were transfected with either pBJ5-Flag-HDAC1 or pME18s-HDAC2 along with or without pSG-myc-Cabin1. Cell lysates were prepared with a lysis buffer consisting of 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, and 1 mM PMSF. HDACs were immunoprecipitated with an anti-Flag antibody (Sigma) and probed with anti-c-myc antibody (Babco). Total HDACs and Cabin1 were detected by Western blot of whole-cell lysates.

(B) Cabin1 recruits mSin3A. Jurkat T cell lysate containing c-myc-tagged Cabin1 was mixed with in vitro transcription/translation product of [^{35}S]mSin3A. Cabin1 was immunoprecipitated with an anti-c-myc antibody, and bound [^{35}S]mSin3A was visualized by autoradiography after SDS-PAGE.

(C) Coimmunoprecipitation of endogenous Cabin1 and endogenous HDAC1 and 2. Nuclear extracts of DO11.10 T cells were prepared as described previously (Sun et al., 1998). Nuclear extracts (0.5 mg/lane), prepared from DO11.10 T cells (Sun et al., 1998), were incubated with anti-HDAC1, anti-HDAC2, or anti-HDAC4 polyclonal antibodies for 12 hr, followed by immunoprecipitation, SDS-PAGE, and Western blotting using anti-Cabin1 antibodies.

amino acid fragment is sufficient for mediating the Cabin1-mSin3A interaction. The MAD family of corepressors, N-CoR, and SMRT are known to interact with mSin3 via a short Sin3 interacting domain (SID) (Ayer et al., 1995; Hurlin et al., 1995; Schreiber-Agus et al., 1995; Alland et al., 1997; Heinzel et al., 1997; Nagy et al., 1997). Sequence comparison between SID sequences from those corepressors and the SID-containing N-terminal domain of Cabin1, however, did not reveal significant similarity, suggesting that Cabin1 is likely to employ a different type of SID for mSin3 interaction. More detailed mapping will be required to define the minimal SID domain in Cabin1.

mSin3A contains four paired amphipathic helix (PAH) domains, a subset of which has been shown to mediate interactions between mSin3A and other corepressors. We determined the domain in mSin3A that mediates its interaction with Cabin1 by coimmunoprecipitation using mSin3A truncation fragments and Cabin1 expressed in Jurkat T cells. Deletions of PAH4, the HDAC-interacting domain (HID), and PAH3 have no effect on the binding of mSin3A to Cabin1 (Figures 3C and 3D). However, deletion of the PAH2 domain (N205) abolished the interaction between Cabin1 and mSin3A, indicating that the PAH2 domain is required for binding of mSin3A to Cabin1. This result is similar to the MADS family of corepressors that also bind to mSin3 through the PAH2 domain (Ayer et al., 1995; Hurlin et al., 1995; Schreiber-Agus et al., 1995). However, this situation is different from N-CoR, which binds to the PAH3 and PAH1 domains of mSin3 through the N and C termini, respectively (Alland et al., 1997; Heinzel et al., 1997). The lack of sequence similarity between SIDs in the MAD family of corepressors and the NH_2 fragment of Cabin1, despite their shared binding to PAH2 in mSin3A, suggests that PAH2 is capable of interacting with different types of protein domains.

Association of HDAC1 and 2 with Cabin1 Accounts for Most of Cabin1-Mediated Repression of MEF2

To assess whether HDACs bound by Cabin1 and MEF2 are enzymatically active, we coexpressed Cabin1, mSin3A, HDAC1, and MEF2D in Jurkat T cells and determined whether the HDAC activity remained associated with each protein after they are immunoprecipitated from cell lysate. Acetylated histones purified from sodium butyrate-treated Jurkat cells (Carmen et al., 1996) were used as substrates. The deacetylation reaction was followed using an anti-acetylated lysine antibody that specifically recognized acetylated but not unacetylated H4 histone. HDAC activity was detected in the immunoprecipitate of Cabin1 as well as mSin3 and anti-HDAC1 antibodies. As Cabin1 has been shown to bind to MEF2 in a Ca^{2+}-sensitive fashion, it was predicted that association between MEF2 and HDACs should exhibit similar sensitivity to Ca^{2+}. Indeed, when MEF2D was immunoprecipitated, HDAC activity was detected only in the presence of EGTA but not in the presence of Ca^{2+} (Figure 4A). In contrast, the interaction between Cabin1 and mSin3A is not sensitive to the concentration of Ca^{2+} (H.-D. Y. and J. O. L., unpublished data). Thus, the Ca^{2+}-dependent "switch" of the association between MEF2 and HDACs is conferred at the level of Cabin1-MEF2 interaction.

One important feature shared by all known transcription corepressors is their ability to repress transcription

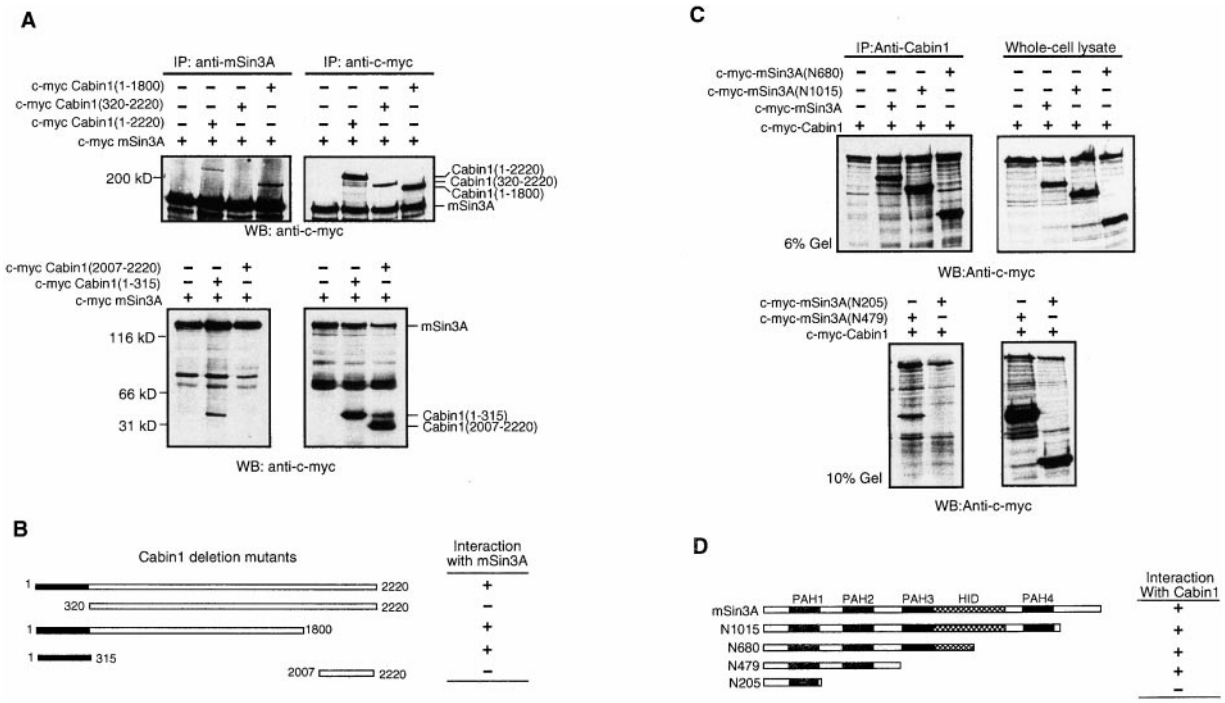

Figure 3. Mapping of Cabin1-mSin3A Interacting Domains

(A) Coimmunoprecipitation of Cabin1 truncation mutants with mSin3A. Jurkat T cells were transfected with various c-myc-tagged Cabin1 truncation mutants along with c-myc-tagged pCS2 + MT-mSin3A. Cell lysates were immunoprecipitated with either anti-mSin3A antibody (Santa Cruz) or anti-c-myc antibody and probed with anti-c-myc antibody.

(B) Schematic representations of Cabin1 truncation mutants and their interaction with mSin3A. The mSin3A interacting domains are highlighted by closed bars.

(C) Coimmunoprecipitation of mSin3A truncation mutants with Cabin1. Jurkat T cells were transfected with pCS2 + MT encoding various c-myc epitope–tagged mSin3A truncation mutants along with c-myc-tagged Cabin1. Cell lysates were immunoprecipitated with polyclonal anti-Cabin1 antibodies. The expression level of each protein was detected by probing 10% of cell lysates with anti-c-myc antibody.

(D) Schematic representations of various mSin3A deletion mutants and their interaction with Cabin1.

of constitutively active promoters when recruited to their vicinity (Alland et al., 1997; Hassig et al., 1997; Heinzel et al., 1997; Kadosh and Struhl, 1997; Laherty et al., 1997; Zhang et al., 1997). We thus expressed a fusion protein between the Gal4 DNA binding domain and the full-length Cabin1 and determined the ability of the resultant fusion protein to repress a constitutively active reporter gene placed in close proximity to multimerized Gal4 binding sites. Cabin1 indeed repressed the reporter gene activity by over 6-fold (Figure 4B), confirming that Cabin1 is capable of silencing a constitutively active promoter when it is recruited to the vicinity of the promoter. We then used this transcriptional repression assay to determine the domain in Cabin1 that is required for mediating transcriptional repression. While the N-terminal Cabin1(1–315) retained the repressive activity of the full-length Cabin1, the C-terminal fragment, Cabin1(2007–2220), had no effect on the reporter gene activity (Figure 4B). Thus, the N-terminal 315 amino acid fragment is sufficient for mediating transcriptional repression by Cabin1.

To further assess the role of HDACs in Cabin1-mediated transcriptional repression, we determined the effect of trichostatin A (TSA), an HDAC inhibitor (Yoshida et al., 1990; Taunton et al., 1996), on the MEF2-dependent reporter gene activation induced by PMA and ionomycin. TSA significantly enhanced the MEF2 reporter gene activity in a concentration-dependent manner (Figure 4C). Expression of Cabin1 led to a dose-dependent resistance to both the combination of PMA and ionomycin and TSA. These results are consistent with the notion that Cabin1 is a corepressor of MEF2 and its major mechanism of transcriptional repression is through recruitment of mSin3 and HDACs.

Competition of Cabin1 against p300 for MEF2 Constitutes a Second Mechanism of Transcriptional Repression By Cabin1

Although the N-terminal 315 amino acid fragment of Cabin1 is both necessary and sufficient for binding to mSin3A (Figures 3A and 3B) and for repression of a 4xGal4–14D luciferase reporter gene (Figure 4B), the C-terminal fragments containing the minimal MEF2 binding domain such as Cabin1(2154–2220) can still inhibit MEF2-dependent transcription, albeit with a lower potency than that for the full-length Cabin1 (Youn et al., 1999). Thus, Cabin1 may repress MEF2 transcriptional activity by another mechanism. MEF2 is known to bind the coactivator p300 (Sartorelli et al., 1997). Like Cabin1, p300 also binds to MEF2 through its N-terminal MADS/MEF2 box, raising the possibility that Cabin1 and p300 may bind to MEF2 in a mutually exclusive manner. p300 is known to bind transcription activators via either its N-terminal or C-terminal domains. When Flag-p300

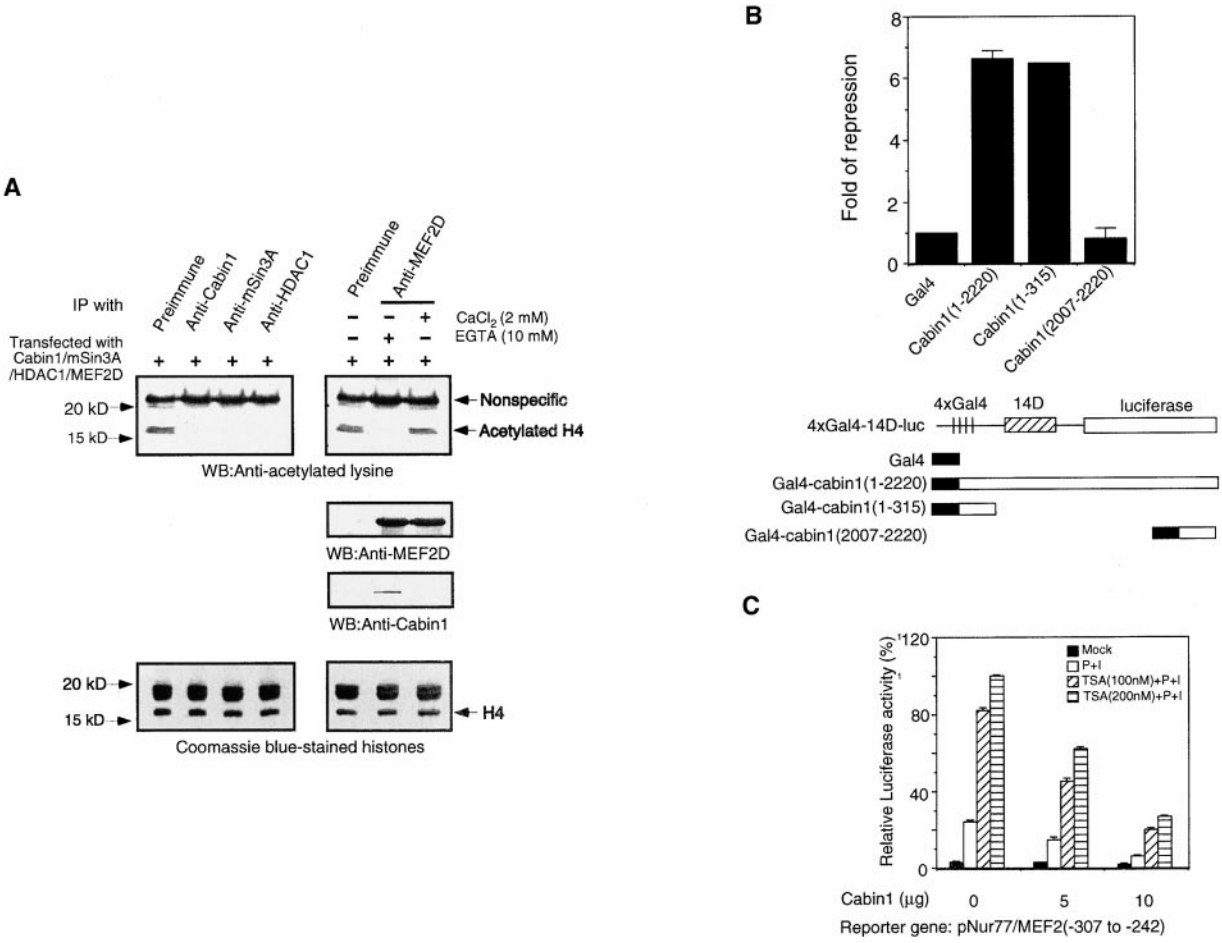

Figure 4. Cabin1 Represses MEF2D Transcriptional Activity by Recruiting HDACs

(A) MEF2 is associated with histone deacetylase activity in calcium-sensitive manner. Jurkat T cells were transfected with Cabin1, mSin3A, HDAC1 and MEF2D. Cell lysates were prepared with normal lysis buffer or lysis buffer containing either EGTA or $CaCl_2$ and immunoprecipitated with antibodies as indicated. Immunoprecipitates were washed twice with lysis buffer and twice with the HD buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, and 10% glycerol). One microgram of histones purified from sodium butyrate-treated Jurkat T cells (Carmen et al., 1996) was added to each of immunoprecipitates, with the total volume adjusted to 30 μl in HD buffer. The mixtures were incubated at 37°C for 4 hr before they were subjected to an 18% SDS-PAGE followed by Western blot with an anti-acetylated lysine antibody (Upstate). This antibody appeared to be selective for acetylated histone H4, as other histone isoforms cannot be detected at this level of total histone used.

(B) Repression of the 4xGal4–14D-luciferasse reporter gene activation by Gal4-Cabin1. Jurkat cells were transfected with 15 μg Gal4-Cabin1 and its deletion mutants together with 5 μg 4xGal4–14D-luciferase reporter plasmid. Fold repression was calculated by comparing reporter gene activities with that resulting from the Gal4 vector.

(C) Trichostatin A reverses the repressional effect of Cabin1 on the MEF2D reporter. Jurkat cells were transfected with varying amounts of pSG-Cabin1 and the transfected cells were allowed to recover for 6 hr. They were then treated with TSA (Calbiochem) for 1 hr before an overnight stimulation with PMA (40 nM) and ionomycin (1 μM). Luciferase activity was normalized to β-galactosidase activity.

(1–682) and Flag-300(1737–2414) were coexpressed with MEF2D, both fragments coimmunoprecipitated with MEF2D with the C-terminal p300(1737–2414), exhibiting significantly higher affinity for MEF2D (Figure 5A). To determine whether Cabin1 and p300 compete for MEF2, we expressed Flag-p300, c-myc-Cabin1, and MEF2D individually in Jurkat T cells, prepared each lysates in the presence of either EGTA or $CaCl_2$, and mixed them before determining the binding of these proteins by coimmunoprecipitation. In the presence of EGTA, MEF2D is bound predominantly to Cabin1 with a small amount of p300, likely due to its overexpression. In the presence of Ca^{2+}, however, MEF2D is bound almost exclusively to p300 with no detectable Cabin1 (Figure 5B). The mutually antagonistic relationship between Cabin1 and p300 for MEF2 binding was further underscored by the opposite effects they exert on a MEF2-dependent reporter gene activation in vivo (Figure 5C). While p300 enhanced the MEF2 reporter gene activation in a dose-dependent manner, coexpression of Cabin1 decreased the reporter gene activity in a dose-dependent fashion. The competitive binding of Cabin1 and p300 to MEF2 thus mediates the Ca^{2+}-dependent inactivation and activation of MEF2 transcriptional activity, respectively.

Calcium Signaling Regulates MEF2 through Both the N-Terminal MADS/MEF2 Domain and the C-Terminal Transactivation Domain

We have previously shown that the interaction between Cabin1 and MEF2 through its DNA binding domain is

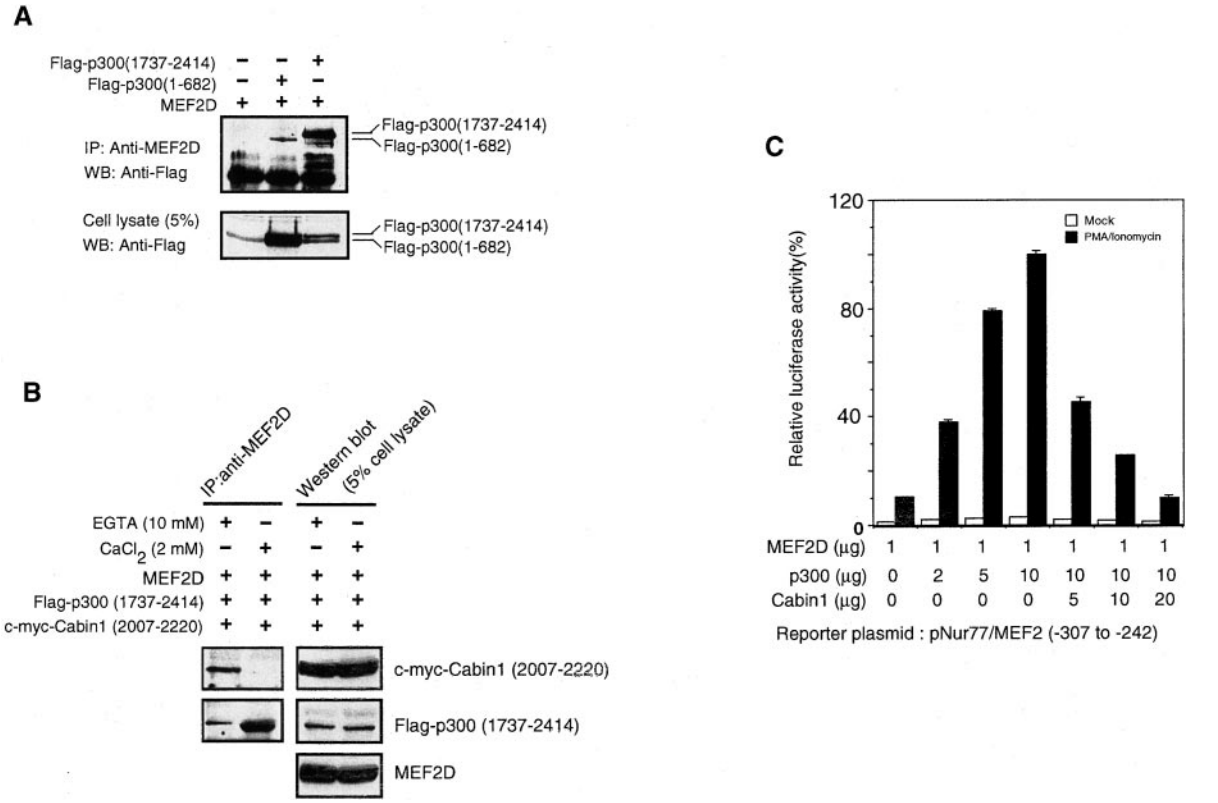

Figure 5. Competition between Cabin1 and p300 for MEF2 Binding

(A) MEF2 strongly binds to the C-terminal domain of p300. Jurkat T cells were transfected with either Flag-tagged p300(1–682) or p300(1737–2414) along with pSG-MEF2D. Cell lysates were subjected to immunoprecipitation with an anti-MEF2D antibody and probed with an anti-Flag antibody.

(B) Specific binding of MEF2D with either Cabin1 or p300 in a calcium-dependent manner. Plasmids encoding MEF2D, p300(1737–2414), and Cabin1(2007–2220) were transfected into Jurkat cells separately. Each cell lysate was prepared with a lysis buffer containing either EGTA or $CaCl_2$, incubated on ice for 1 hr before they were mixed, and incubated at 4°C for 2 hr to allow protein association. MEF2D was immunoprecipitated and subjected to SDS-PAGE followed by Western blot using anti-c-myc antibody or anti-Flag antibody.

(C) Mutually antagonistic effects of Cabin1 and p300 on the transcriptional activity of MEF2D. Jurkat cells were transfected with indicated amounts of Cabin1(FL) and p300(FL) along with Nur77/MEF2D(−307 to −242) luciferase reporter plasmid. After a 24 hr recovery period, transfected cells were treated overnight with PMA and ionomycin. Luciferase activity was normalized to β-galactosidase activity.

regulated by calcium (Youn et al., 1999). It remained to be determined whether calcium signaling also affects the activity of the transactivation domain of MEF2. To address that question, we made several constructs expressing different fusion proteins and determined their transcriptional activity using appropriate reporter genes (Figure 6). Whereas the activation of the Gal4-luciferase reporter gene by the Gal4-VP16 fusion protein is further enhanced by treatment with PMA and ionomycin, this activation is insensitive to treatment with the calmodulin antagonist trifluoperazine (TFP) (Kahn et al., 1998). In contrast, activation induced by fusion proteins between either the full-length MEF2D or the transactivation domain of MEF2D and Gal4 DNA binding domain in the presence of PMA and ionomycin is inhibited by TFP, suggesting that transactivation by the MEF2D transactivation domain is also regulated by calcium signal. Importantly, the activation of a MEF2 reporter gene mediated by a fusion protein between the N-terminal MADS-MEF2 domain of MEF2C and the VP-16 activation domain is also sensitive to inhibition by TFP, consistent with our previous finding that the association between the N-terminal MADS/MEF2 domain and Cabin1 is sensitive to calcium signaling.

Discussion

TCR-induced expression of Nur77 family of proteins leads to thymocyte apoptosis. As a repressor of MEF2, a calcium-dependent transcription factor mediating Nur77 transcription, Cabin1 is responsible for keeping the Nur77 promoter silent in the absence of a TCR signal. This repression has been shown to be released by the second messenger calcium in response to TCR signaling (Youn et al., 1999). We showed that MEF2 is constitutively bound to the Nur77 promoter regardless of the activation status of T cells. In unactivated T cells, MEF2 is bound to a transcriptional repression complex consisting of Cabin1, mSin3, and HDAC1 and HDAC2. Upon TCR signaling and calcium influx, activated calmodulin binds to Cabin1, releasing it from MEF2, vacating the MADS/MEF2 domain for association with the coactivator p300 (Figure 7). Thus, the Ca^{2+}-dependent association and dissociation of two opposing classes of chromatin remodeling enzymes are responsible for tight control of Nur77 transcription, ensuring that thymocytes will not commit to apoptosis in the absence of TCR signaling.

Remodeling of chromatin by addition and removal of

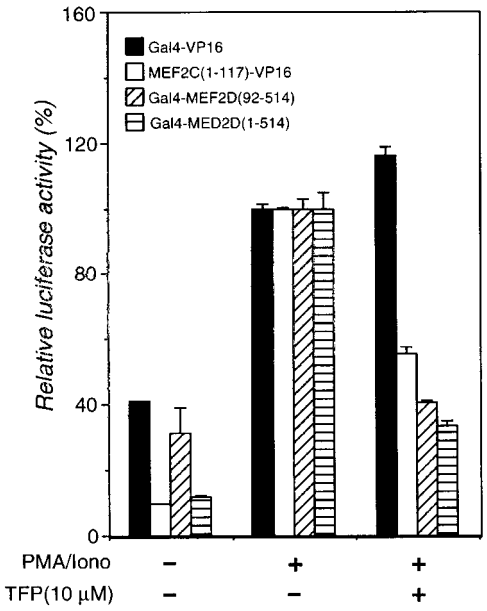

Figure 6. Calcium Signaling Activates MEF2 through both the DNA Binding Domain (MADS/MEF2 box) and the Transactivation Domain

Jurkat cells were transfected with Gal4-MEF2D(1–514), Gal4-MEF2D(92–514), and Gal4-VP16 along with pG5-luciferase reporter, or MEF2C(1–117)-VP16 along with pNur77(−307 to −242)-luciferase reporter. Each transfected cell was pretreated with 10 μM trifluoperazine (TFP) for 1 hr, followed by incubation with 40 nM PMA and 1 μM ionomycin overnight. Luciferase activity was normalized to β-galactosidase activity.

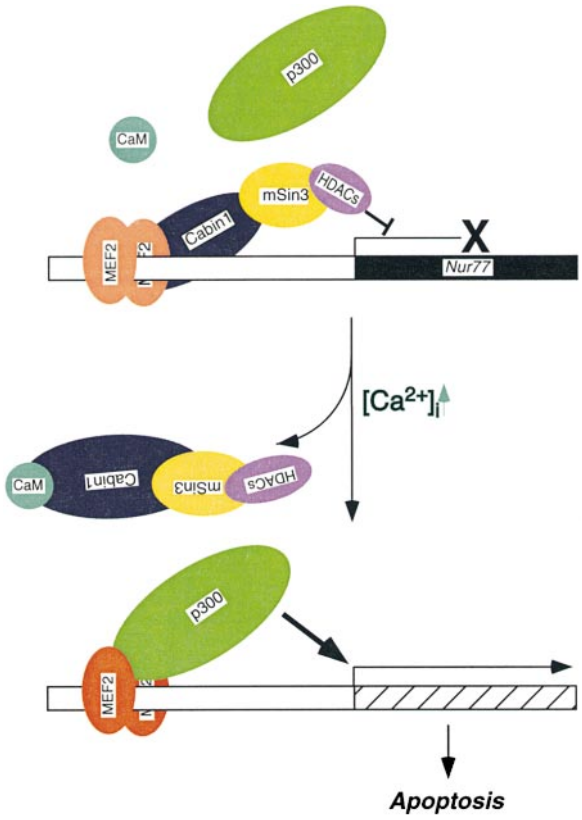

Figure 7. A Model of Calcium-Dependent Recruitment of the p300 HAT and HDACs by Cabin1 Mediates MEF2 Activation in Response to Ca^{2+} Signal

Note that other calcium-dependent signaling pathways impinging on MEF2, including calcineurin and calmodulin-dependent kinases, are not shown.

acetyl groups from the core histones has recently been shown to be an important mechanism for regulation of transcription. A picture of how different signal transduction pathways impinge upon histone acetylases (HATs) and HDACs to regulation transcription has begun to emerge. Our studies on the regulation of the MEF2 family of transcription factors revealed both common and distinct features for the control of MEF2 by Cabin1 in comparison with other known corepressors. Similar to other corepressors such as MAD (Hassig et al., 1997; Laherty et al., 1997; Zhang et al., 1997), N-CoR (Alland et al., 1997; Heinzel et al., 1997; Nagy et al., 1997), and Rb (Brehm et al., 1998; Magnaghi-Jaulin et al., 1998), repression of MEF2 by Cabin1 is mediated in large part by recruitment of mSin3 and HDACs. In addition to recruitment of the mSin3/HDAC1–2 complexes, we uncovered a second mechanism by which Cabin1 represses MEF2; it binds to MEF2 at the same domain that mediates p300 binding. The binding of Cabin1 thus not only recruits HDACs to remodel chromatin into a transcriptionally inactive state, but it also blocks the binding of the p300 HAT. In this respect, Cabin1 bears similarity to the recently reported Smad corepressor TGIF, which recruits the mSin3 complex and whose binding to Smad2 is mutually exclusive with p300 (Wotton et al., 1999).

We have shown that Cabin1-mediated HDAC recruitment to MEF2 is dependent on calcium. This was further confirmed using the MEF2C(1–117)-VP16 fusion protein; its activation by PMA and ionomycin is sensitive to inhibition by the calmodulin antagonist TFP. As only the N-terminal MADS/MEF2 domain is present in this fusion protein, the calcium-dependent activation of MEF2C (1–117)-VP16 can only be attributed to regulation of Cabin1-MEF2 interaction. In addition, we also determined whether the transactivation domain of MEF2 is also subject to calcium regulation. Indeed, the activity of Gal4-MEF2D(92–514) was found to be sensitive to calcium (Figure 6), suggesting that the transactivation of MEF2 is also regulated by calcium. Thus, MEF2 is regulated by calcium signaling through multiple pathways.

Cabin1/Cain was originally identified as an endogenous inhibitor of calcineurin and shown to play a negative regulatory role in calcineurin-mediated signal transduction pathways (Lai et al., 1998; Sun et al., 1998). We have now identified a second function of Cabin1 as a transcriptional repressor of MEF2 involved in TCR-mediated Nur77 expression and T cell apoptosis (Youn et al., 1999). Whether the calcineurin binding domain of Cabin1 plays a role in TCR-mediated Nur77 expression remains to be explored.

In addition to Cabin1, two other MEF2 corepressors were reported recently; they are the MEF2 interacting transcriptional repressor (MITR) and HDAC4 (Miska et al., 1999; Sparrow et al., 1999). Similar to Cabin1, both MITR and HDAC4 bind to the MADS/MEF2 box of MEF2. Unlike Cabin1, however, neither MITR nor HDAC4 has been found to bind to MEF2 in a calcium-sensitive manner (Miska et al., 1999; Sparrow et al., 1999). Moreover,

the conserved N-terminal MEF2 binding domains of MITR and HDAC4 had little effect on MEF2-dependent reporter gene activity, suggesting that MITR and HDAC4 may be bound to the MADS/MEF2 box at a site different from that for Cabin1 binding. This result is consistent with the lack of significant sequence similarity between the minimal MEF2 binding domains of MITR or HDAC4 and Cabin1. Based on limited tissue distribution studies at the mRNA level, Cabin1, MITR, and HDAC4 appear to have largely distinct patterns of tissue distribution, with overlap in certain tissues (Sun et al., 1998; Grozinger et al., 1999; Sparrow et al., 1999). Of relevance to TCR-mediated Nur77 and Nor1 expression, all three MEF2 corepressors were found at significant levels in thymus, suggesting that they play somewhat redundant functions in thymocytes. Whether all three repressors of MEF2 are relevant in silencing Nur77 promoter remains to be investigated. What appears to distinguish Cabin1 from all other known transcription corepressors is its ability to bind to calmodulin, which is accompanied by its dissociation from MEF2. The Ca^{2+}, calmodulin–dependent binding of Cabin1 to MEF2 allows for the coupling of MEF2 transcriptional activity to calcium signaling (Figure 7).

Since the submission of this manuscript, we have finished another study extending the findings on Cabin1 to the growing family of MEF2 repressors including HDAC4, 5, and 7 and MITR (Youn et al., 2000). During the same period, a few reports appeared that describe possible roles of calcineurin and calmodulin-dependent kinases in the regulation of MEF2 (Blaeser et al., 2000; Lu et al., 2000; Wu et al., 2000). It thus appears that multiple calcium signaling pathways are involved in the regulation of MEF2 activity and possibly Nur77 expression.

Experimental Procedures

Cell Culture

Jurkat T cells and DO11.10 T hybridoma cells were grown in RPMI medium supplemented with 10% serum, 2 mM glutamine, and 50 U/ml streptomycin/penicillin.

Transfection and Plasmids

T cells were transiently transfected by electroporation (250V, 950 μF) as described previously (Sun et al., 1998). Cabin1 deletion mutants were subcloned into pSG5 mammalian expression vector (Stratagene). Gal4-fused Cabin1-deletion mutants were subcloned into pM vecter (Clontech). Other reagents were generously provided by the following scientists: Flag-tagged HDAC1 and 2, E. Seto (University of South Florida); c-myc tagged mSin3A deletion mutants and 4xGal4–14D-luc reporter, Robert Eisenman (Fred Hutchinson Cancer Research Center); T7-mSin3A, D. Ayer (University of Utah); Flag-tagged p300 deletion mutants, Tetsuya Taga (Tokyo Medical and Dental University); and HA-tagged p300, Yang Shi (Harvard Medical School). More detailed information about plasmids used in this study is available upon request.

Immunoprecipitation

T cells were lysed with a lysis buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 0.5% NP-40, and 1 mM PMSF) and incubated with suitable antibodies and protein A/G beads for 2 hr at 4°C. The beads were then washed three times in lysis buffer. Immunoprecipitates were subjected to SDS-PAGE and probed with appropriate antibodies. Anti-HDAC1 and anti-mSin3A antibodies were purchased from Santa Cruz, anti-flag antibody from Sigma, anti-c-myc antibody from Babco, anti-MEF2D from R. Prywes (Columbia University), and anti-HDAC1, anti-HDAC2, and anti-HDAC4 antibodies from S. Schreiber (Harvard University).

HDAC Assay

Acetylated histones were purified from Jurkat T cells as described previously (Carmen et al., 1996). Jurkat cells (5×10^7) were incubated with 10 mM sodium butyrate for 1 hr, harvested, and washed once with PBS containing 10 mM sodium butyrate. The cells were lysed in a lysis buffer consisting of 20 mM Tris-HCl [pH 7.4], 100 mM NaCl, 10 mM sodium butyrate, 1% NP-40, and 1mM PMSF, and washed twice with the same buffer. Sulfuric acid (0.2 M) was added to packed nuclei and incubated for 90 min with stirring at 4°C. Ten volumes of chilled acetone were added to the supernatant and the resulting mixture was kept at −20°C overnight. Precipitates were collected by centrifugation, dried, and dissolved in water to a final concentration of 3 mg/ml.

CHIP Assays

CHIP assays were carried out as described previously (Braunstein et al., 1993; Luo et al., 1998). Jurkat T cells were transfected with 5 μg pSGMEF2D, 10 μg pSGCabin1, with or without 5 μg pGLNur77 (−307 to −242)-luc. After incubation at 37°C for 24 hr, cells were treated with 1% formaldehyde for 15 min at 37°C, lysed in a buffer consisting of 5 mM PIPES (pH 8.0), 85 mM KCl, and 0.5% NP-40. The nuclei were precipitated and lysed in a lysis buffer (50 mM Tris-HCl [pH 8.1], 10 mM EDTA, 1% SDS, 1 mM PMSF). Nuclear lysates were sonicated and diluted 10-fold with an IP buffer (16.7 mM Tris-HCl [pH 8.1], 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS, and 1.1% Triton X-100). Lysates were incubated with either anti-MEF2D or anti-Cabin1 antibodies for 2 hr at 4°C. Immunoprecipitates were washed three times with wash buffer and twice with TE buffer. Immune complexes were eluted with 2 vol of 250 μl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) and 20 μl of 5 M NaCl was added to reverse formaldehyde cross-linking. DNA was extracted with phenol/chloroform and precipitated with ethanol. Precipitated DNA was resuspended with water and amplified by PCR with primers corresponding to the flanking regions of the MEF2 binding sites on the Nur77 promoter in the pGL2 basic vector (Promega).

In Vitro Transcription/Translation

^{35}S-labeled mSin3A was made using a STP3(T7) in vitro transcription/translation kit (Novagen).

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