The Modular Nature of Histone Deacetylase HDAC4 Confers Phosphorylation-dependent Intracellular Trafficking*

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In C2C12 myoblasts, endogenous histone deacetylase HDAC4 shuttles between cytoplasmic and nuclear compartments, supporting the hypothesis that its subcellular localization is dynamically regulated. However, upon differentiation, this dynamic equilibrium is disturbed and we find that HDAC4 accumulates in the nuclei of myotubes, suggesting a positive role of nuclear HDAC4 in muscle differentiation. Consistent with the notion of regulation of HDAC4 intracellular trafficking, we reveal that HDAC4 contains a modular structure consisting of a C-terminal autonomous nuclear export domain, which, in conjunction with an internal regulatory domain responsive to calcium/calmodulin-dependent protein kinase IV (CaMKIV), determines its subcellular localization. CaMKIV phosphorylates HDAC4 in vitro and promotes its nuclear-cytoplasmic shuttling in vivo. However, although 14-3-3 binding of HDAC4 has been proposed to be important for its cytoplasmic retention, we find this interaction to be independent of CaMKIV. Rather, the HDAC4:14-3-3 complex exists in the nucleus and is required to confer CaMKIV responsiveness. Our results suggest that the subcellular localization of HDAC4 is regulated by sequential phosphorylation events. The first event is catalyzed by a yet to be identified protein kinase that promotes 14-3-3 binding, and the second event, involving protein kinases such as CaMKIV, leads to efficient nuclear export of the HDAC4:14-3-3 complex.

Accumulating evidence indicates that active transcriptional repression is an important component of many physiological events regulated at the level of gene expression, including muscle differentiation (1). The repression of transcription is manifest at the level of chromatin structure where histone deacetylases (HDACs) are recruited to deacetylate histones and create a repressive chromatin structure (reviewed in Ref. 2). Of the ten human HDACs identified so far (3), HDAC4 and its closely related family member HDAC5 have been specifically implicated in regulating muscle differentiation ((1) and see below).

The functional link between HDAC4/5 and muscle differentiation was first uncovered by the cloning of MITR, a transcriptional repressor identified as an interactive partner for myocyte enhancer factor 2 (MEF-2) transcription factor family members, which are important for muscle differentiation (4). MITR shows extensive homology to the non-catalytic N terminus of HDAC4 and -5 (4). Indeed both HDAC4 and HDAC5 interact with MEF-2. It was reported that overexpression of HDAC4 or HDAC5 represses MEF-2 transcriptional activity (5) and suppresses C2C12 myoblast differentiation (1). It was also found that the HDAC4/5-MEF-2 interaction and the effect of this complex on muscle differentiation could be reversed by a constitutively active form of a calcium/calmodulin-dependent protein kinase (CaMK) (6). However, the mechanism by which CaMK regulates HDAC4 and HDAC5 is not entirely clear.

When ectopically expressed, HDAC4 can be found in either the nucleus or cytoplasm whereas the closely related HDAC5 is predominantly a nuclear protein (7) and see Fig. 1). The observed localization patterns of different HDACs support the idea that the deacetylase activity of HDAC4 and HDAC5 might be controlled by their differential distribution in subcellular compartments and suggest that subcellular trafficking may be dynamically regulated. Indeed, inhibition of the nuclear export machinery leads to nuclear accumulation of HDAC4, indicating that its subcellular localization is controlled by active nuclear export (7). However, nuclear export activity alone is not likely sufficient to explain the intracellular trafficking of HDAC4, because not all cells expressing HDAC4 show identical distribution. This suggests the presence of other regulatory mechanisms required for the control of HDAC4 subcellular localization. One such mechanism may involve members of the 14-3-3 protein family, which bind a consensus motif that contains specific phosphorylated serine residues (8). HDAC4 binds 14-3-3 family members, and the mutation of three serine residues that abolishes 14-3-3 binding leads to nuclear accumulation of HDAC4. Based on such studies, it was proposed that 14-3-3 traps HDAC4 in the cytoplasm (9, 10). More importantly, the association with 14-3-3 is sensitive to phosphatase inhibitors, suggesting that this interaction, and therefore the subcellular distribution of HDAC4, is regulated by reversible phosphorylation (9). This idea is supported by the observation that the ability of HDAC4 to repress transcription can be inhibited by active forms of CaMK (6). Together, these observations suggest the possibility that subcellular localization of HDAC4

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might be regulated by CaMK-dependent phosphorylation.

Although the analysis of ectopically expressed HDAC4 suggests that its subcellular localization is regulated by nuclear export, this has not been shown for the endogenous protein. In this report, we present evidence that endogenous HDAC4 shuttles between nuclear and cytoplasmic compartments during muscle differentiation, suggesting a dynamic regulation of HDAC4 by differential subcellular localization. Furthermore, we show that subcellular localization of HDAC4 is controlled by both a C-terminal autonomous export domain and a central regulatory domain. We further show that the expression of an active form of CaMKIV mobilizes HDAC4 from nucleus to cytoplasm and that HDAC4 can be phosphorylated by CaMKIV in vitro, suggesting that CaMKIV-dependent phosphorylation can control HDAC4 subcellular localization. We present evidence that HDAC4 constitutively binds 14-3-3 and this interaction is necessary for HDAC4 to respond to CaMKIV. However, CaMKIV promotes HDAC4 nuclear export without stimulating HDAC4-14-3-3 interaction. These results suggest the existence of two separate phosphorylation events in which HDAC4 is first phosphorylated by a yet to be identified kinase that creates an HDAC4-14-3-3 complex, followed by secondary phosphorylation events, catalyzed by kinases such as CaMKIV that activate HDAC4 nuclear export.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs and Antibodies**—FLAG-tagged PB35-HDAC4 and HDAC5 expression plasmids were kindly provided by Dr. S. Schreiber (Harvard University). pB/S-HDAC4-S467A/S632A (25/SA) mutant was generated using a QuikChange site-directed mutagenesis kit (Stratagene). Constitutively active and inactive CaMKIV constructs were previously described (11). 3X MEF-2 luciferase reporter construct was kindly provided by Dr. Eric Olson (University of Texas Southwestern Medical Center). Gal4 DNA binding domain (Gal4DBD)-HDAC4 fusion constructs were generated in pCMX-Gal4 vector (12). Retrovirus-mediated gene transfer and analysis of pools of infected cell lines, retroviruses expressing HDAC4, or HDAC5 during muscle differentiation, we established C2C12 cell lines that stably express FLAG-tagged HDAC4 or HDAC5. We stably introduced HDAC4 or HDAC5 by retrovirus-mediated gene transfer and analyzed pools of infected C2C12 cells to avoid potential artifacts that might be associated with a small number of selected clones. We then followed the subcellular distribution of HDAC4 and HDAC5 in infected C2C12 myoblasts by immunohistochemistry using antibodies against HDAC4, HDAC5, or the FLAG epitope. C2C12 cells that stably express ectopic HDAC4 or HDAC5 display no gross abnormalities. As shown in Fig. 1E, HDAC4 is found to be more concentrated in the cytoplasm of the dividing undifferentiated myoblasts. This cytoplasmic population of HDAC4 responds to LMB treatment and is retained in the nucleus (data not shown). In contrast, a majority of HDAC5 is already localized in the nucleus of the cycling C2C12 cells (Fig. 1H). These results suggest that the subcellular localization of HDAC4 and HDAC5 may be regulated differently. To determine if HDAC4 translocates into nucleus upon terminal differentiation, we induced differentiation of myoblasts stably expressing HDAC4 or HDAC5. As shown in Fig. 1I (F and G), strong nuclear staining of HDAC4 is observed in the differentiated myoblasts (arrowheads). Similar results were obtained using another IOD-3-3 antibody. These observations support the idea that a portion of HDAC4 accumulates in the nucleus upon differentiation. In fact, in some C2C12 cells, nuclear accumulation of HDAC4 can be observed 24 h after the differentiation stimulus (data not shown). On the other hand, HDAC5 remains concentrated in the nuclear compartment in the differentiated myoblasts (Fig. 1, J and J). Together, our results demonstrate a relocation of HDAC4 from cytoplasm to nucleus during muscle...
cell differentiation, whereas the majority of HDAC5 appears to reside in the nucleus in both cycling and differentiated myotubes.

To our surprise, ectopic expression of HDAC4 or -5 does not block C2C12 differentiation. The C2C12 cells containing elevated concentrations of HDAC4 or -5 form multinucleated myotubes (Fig. 1, G, J, L, and M) that express the appropriate terminal differentiation marker MHC (Fig. 1, G and J). In fact, C2C12 cells that stably express HDAC4 or -5 differentiate into large myotubes that contract vigorously. This contraction often leads to their dislodgment from the culture dishes, and the partially detached myotubes become ball-like structures (Fig. 1M, arrowheads). Such structures were never observed in control C2C12 myotubes (data not shown). These observations not only suggest that a portion of HDAC4 translocates to the nucleus in response to differentiation but also raise the possibility that nuclear HDAC4 and/or HDAC5 may play roles in facilitating certain aspects of muscle differentiation.

**HDAC4 Contains an Autonomous Nuclear Export Domain**—The analysis of HDAC4 subcellular localization in C2C12 cells demonstrates that the movement of HDAC4 occurs during muscle differentiation. To examine the structural basis and potential factors that might regulate this movement, we turned to U2OS cells, which are more amenable to structure-function analysis of HDAC4 expressed by transfection. When U2OS cells are cultured in 10% CO₂, transfected HDAC4 is localized in nuclear or cytoplasmic compartments while transfected HDAC5 is exclusively localized in the nucleus (Fig. 2A). Similar to endogenous HDAC4 in myoblasts, treatment with the nuclear export inhibitor LMB leads to nuclear accumulation of the transfected HDAC4 (Fig. 2A, right panel). Thus, this assay system allows us to study the regulation of intracellular HDAC4 shuttling.

We first investigated which region of HDAC4 is required for nuclear export. We fused a series of HDAC4 truncation mutants to the DNA binding domain of the yeast Gal4 transactivation factor (Gal4DBD, Fig. 2B), which contains an intrinsic nuclear localization signal and itself localizes to the nucleus (data not shown). Analysis of the subcellular localization of these Gal4DBD-HDAC4 fusion proteins shows that amino acids 859–1084 at the C terminus of HDAC4 are sufficient to confer nuclear export activity and redistribute a significant amount of nuclear Gal4DBD to the cytoplasm (Figs. 2C, middle left panel and 2D, upper panel). Upon LMB treatment, this fusion protein again accumulates in the nucleus demonstrating that the intrinsic export activity resides in fragment 859–1084 (Figs. 2C, middle right panel and 2D, lower panel). Supporting this conclusion, deletion of the C terminus, which encompasses this domain, induces the nuclear localization of HDAC4 and abrogates the LMB response (data not shown). In addition, Gal4DBD fused with the N-terminal portion of HDAC4 (amino acids 1–223 or 222–630) is also not LMB-responsive (data not shown and Fig. 2, C and D, lower panel). Thus, the C terminus of HDAC4 contains a domain that mediates nuclear export.

**A Central Regulatory Domain Is Required for Stimulation of Nuclear Export by CaMKIV**—The observation that phosphatase inhibitors can affect the subcellular distribution of HDAC4 suggests that the localization of HDAC4 should be regulated by...
protein phosphorylation (9). An active form of calcium/calmodulin-dependent protein kinase IV (CaMKIV) has been shown to be capable of regulating the dissociation of MEF-2 from HDAC4 in vitro (6). Because CaMKIV is present in the U2OS cells, we asked whether ectopic CaMKIV could regulate HDAC4 subcellular localization. To test this hypothesis, we co-transfected HDAC4 with either a constitutively active form of CaMKIV or its corresponding kinase-inactive version into U2OS cells (11). As shown in Fig. 3A (b and c), co-expression of active CaMKIV, but not the kinase-inactive mutant, mobilizes nuclear HDAC4 and leads to its accumulation in the cytoplasm. Importantly, the ability of CaMKIV to mobilize HDAC4 is completely abrogated by LMB treatment demonstrating that CaMKIV functions by influencing export rather than inhibiting nuclear import of HDAC4 (Fig. 3A (d)).

To map the regulatory domain in HDAC4 that is responsive to CaMK, we determined the ability of active CaMKIV to mobilize a series of HDAC4 deletion mutants fused to Gal4DBD. As shown in Fig. 3B, deletion of amino acids 1–221 (fragment Gal4-222–630) does not affect CaMKIV responsiveness. A further deletion to amino acid 629 (fragment Gal4-629–1084) eliminates the CaMKIV response, although this fragment contains the nuclear export domain (Fig. 2). This result indicates that amino acids in the 222–630 fragment of HDAC4 might be regulated by CaMKIV. Consistent with the idea that CaMKIV mobilizes HDAC4 by activating its nuclear export (Fig. 3A), this fragment (222–630) alone without the C-terminal export domain (629–1084) is not responsive to CaMKIV. Together, our results suggest that the subcellular localization of HDAC4 is regulated by two separate domains, one of which confers autonomous export activity while the other confers CaMKIV responsiveness.

CaMKIV-dependent Phosphorylation Induces HDAC4 Cytoplasmic Accumulation—To determine if CaMKIV might modulate HDAC4 subcellular localization by direct phosphorylation, we tested whether HDAC4 is a substrate of CaMKIV. As shown in Fig. 4A, the incubation of purified CaMKIV with a recombinant HDAC4 fragment (453–654) that contains part of the putative CaMK-responsive domain results in HDAC4 phosphorylation. A previous study identified three phosphoserine residues mediating HDAC4 and 14-3-3 interaction (9). Two of these serine residues (Ser-467, Ser-632) are located in this domain, and they fit the consensus phosphorylation sequence for CaMKIV (RXX(S/T) (15)). Indeed, mutation of these two serine residues to alanine (S/A mutant) reduces but does not abolish the phosphorylation of HDAC4-(453–654) fragment by CaMKIV in vitro (Fig. 4A). Thus, CaMKIV can phosphorylate HDAC4 at Ser-467 and/or Ser-632 in vitro.

To assess whether phosphorylation of Ser-467 and Ser-632 is important for the CaMKIV responsiveness of HDAC4 in cells, we co-transfected an expression plasmid encoding the HDAC4 2S/A (S467A/S632A) mutant with active CaMKIV and determined its subcellular localization. As shown in Fig. 4, A and C, the response of the 2S/A mutant to CaMKIV is markedly reduced when compared with wild type or mutant HDAC4 with a single CaMKIV consensus site (serine 467) mutated to alanine (data not shown).

To further assess the functional relevance of phosphorylation by CaMKIV on the subcellular distribution of HDAC4, we examined the transcriptional repression activity of wild type...
HDAC4 and its 2S/A mutant in response to the constitutively active CaMKIV. Overexpression of wild type HDAC4 suppresses MEF-2-dependent transcription (Fig. 4D), and this repression is reversed by co-expression of CaMKIV. In contrast to wild type HDAC4, the 2S/A mutant effectively represses MEF-2 activity but it is more resistant to CaMKIV (Fig. 4D). Collectively, our results suggest that CaMKIV reverses the transcriptional repression activity of HDAC4 by stimulating the mobilization of HDAC4 out of the nucleus.

**HDAC4-14-3-3 Interaction Is Not Stimulated by CaMKIV But Is Necessary for the CaMKIV Responsiveness—**Serine 467 and serine 632 residues have been shown to be involved in 14-3-3 binding (9) and appear to be phosphorylated by CaMKIV in vitro (Fig. 4A). We reasoned that CaMKIV might phosphorylate HDAC4 and promote its binding to 14-3-3, resulting in HDAC4 cytoplasmic accumulation. To test this, we determined the interaction between HDAC4 and 14-3-3 in the presence or absence of active CaMKIV. As shown previously, HDAC4 binds 14-3-3 ((9) and Fig. 4E, lane 1). However, co-expression of CaMKIV does not increase HDAC4 binding to 14-3-3 (lane 2). This result suggests that CaMKIV promotes HDAC4 nuclear export by a mechanism other than direct modulation of HDAC4-14-3-3 interaction. Consistent with this conclusion, both nuclear and cytoplasmic localized HDAC4 is bound to 14-3-3 (data not shown).

To further examine the role of 14-3-3 binding in CaMKIV responsiveness, we determined the binding of the 2S/A mutant to 14-3-3. As shown in Fig. 4E, the 2S/A mutant, which has a much reduced response to CaMKIV (Fig. 4, B and C), also shows reduced binding to 14-3-3 (lanes 3 and 4). Consistent with a critical role of 14-3-3 binding in conferring CaMKIV responsiveness, HDAC4 3S/A mutant (S246A/S467A/S632A), which does not bind 14-3-3 (binding-deficient HDAC4), is completely resistant to CaMKIV in both transcriptional repression and cytoplasmic translocation assays (Fig. 4, B–D). These results suggest that the interaction with 14-3-3 is necessary for HDAC4 to be exported in response to active CaMKIV.

**DISCUSSION**

In this study, we provide evidence that two functional domains work in concert to determine the subcellular localization of HDAC4. One domain, which is present in the C terminus of HDAC4, provides a constitutive nuclear export signal. This domain is presumed to tether HDAC4 to the nuclear export receptor, Crm-1. The central regulatory region, which confers responsiveness to CaMKIV, constitutes the second domain critical to HDAC4 subcellular localization. Importantly, both the central regulatory domain and the C-terminal export domain are required for CaMKIV responsiveness. Thus, the cooperation of these two modular domains determines the specific subcellular localization of HDAC4.

We have identified CaMKIV as one protein kinase that can regulate the subcellular localization of HDAC4, at least in U2OS cells that contain this enzyme. We considered two potential mechanisms that could underlie the effects of CaMKIV on the intracellular trafficking of HDAC4. First, as 14-3-3 binding is critical for HDAC4 subcellular localization and two of the residues that can be phosphorylated by CaMKIV (Ser-467 and Ser-632) also mediate 14-3-3 binding, CaMKIV phosphorylation may result in the interaction of the exported HDAC4 to cytoplasmic 14-3-3, which in turn, retains HDAC4 in the cytoplasm. However, we found no evidence that CaMKIV promotes HDAC4 and 14-3-3 binding despite the fact that the interaction with 14-3-3 appears to be critical for HDAC4 to respond to CaMKIV. Second, CaMKIV-mediated phosphorylation of HDAC4 or its associated proteins may promote active nuclear export of HDAC4. Consistent with this idea, the effect of CaMKIV on HDAC4 subcellular localization can be completely abrogated by the LMB-induced inhibition of nuclear export.

The observation that CaMKIV promotes HDAC4 nuclear export by a mechanism other than the 14-3-3 interaction, we propose that CaMKIV reverses the nuclear export of HDAC4. Moreover, our results suggest that CaMKIV promotes HDAC4 nuclear export by a mechanism other than the 14-3-3 interaction.
export without affecting the interaction with 14-3-3 has several implications. First, it argues that, although CaMKIV can phosphorylate HDAC4 on serine residues (467 and 632) that are important for 14-3-3 binding, these are apparently not the major sites phosphorylated by CaMKIV that results in the nuclear export of HDAC4 in cells. Rather, the CaMKIV-independent interaction between HDAC4 and 14-3-3 strongly suggests that HDAC4 is constitutively phosphorylated by one or more yet to be identified kinases. The nuclear HDAC4/14-3-3 complex, which may be the "HDAC4 form" competent to respond to the active CaMKIV, can then be efficiently exported from the nucleus in response to active CaMKIV. Consistent with this idea, both CaMKIV and 14-3-3 can be found in the nucleus (16, 17). Importantly, although 14-3-3 binding appears to be essential for HDAC4 to undergo nuclear-cytoplasmic trafficking in response to CaMKIV, this binding is clearly not required for the nuclear import of HDAC4 and not essential for HDAC4 to function as a transcriptional repressor (Fig. 4D).

It is possible that a CaMKIV-mediated phosphorylation event may promote the interaction of the HDAC4/14-3-3 complex with the Crm-1 export machinery and lead to efficient nuclear export. If HDAC4 is a direct target of CaMKIV, 14-3-3 binding might play a permissive role to render HDAC4 competent to respond to the nuclear export machinery upon HDAC4 phosphorylation, possibly by functioning as a scaffold protein (reviewed in Ref. 18). Consistent with this idea, we note that in vitro, CaMKIV can phosphorylate HDAC4 on the residues other than the two involved in 14-3-3 binding (data not shown). Alternatively, CaMKIV may phosphorylate other proteins important for HDAC4 subcellular localization. For example, it has been reported that CaMKIV can directly phosphorylate MEF-2 (19). It is possible that this phosphorylation event might be sufficient to dissociate the MEF-2-HDAC4 complex and mobilize HDAC4 out of the nucleus. The identification of the "second" phosphorylation event by CaMKIV will be key to substantiate a two-step phosphorylation model and fully understand how HDAC4 subcellular localization is regulated.
Surprisingly, our results reveal a dramatic difference between the subcellular localization of HDAC4 and HDAC5 despite their extensive sequence homology. Even though both HDAC4 and HDAC5 can be regulated by nuclear export, the subcellular localization of HDAC4 and HDAC5 is clearly distinguishable (Fig. 1). Under the identical experimental conditions, the majority of HDAC5 is localized in the nucleus while a significant portion of HDAC4 is localized in the cytoplasm. Although the exact molecular basis underlying this difference is not clear, we have found that, similar to HDAC4, HDAC5 also contains an autonomous export domain. The regulatory domains of HDAC4 and HDAC5, however, could be differentially phosphorylated, which then determine their ability to associate with 14-3-3 and ultimately their respective subcellular localization. Consistent with this hypothesis, although ectopically expressed HDAC5 does not (17, Fig. 4E and data not shown). Recently, it was reported that an active form of calcium/calmodulin-dependent protein kinase I (CaMKI) is capable of promoting HDAC5-14-3-3 binding and leads to HDAC5 export in cultured cells (17). Based on the two-step phosphorylation model that we proposed for HDAC4, it is possible that active CaMKI can play both roles for HDAC5 and induce both 14-3-3 binding as well as nuclear export. Regardless of the details, the different subcellular localization of HDAC4 and -5 suggests that these two closely related proteins may be regulated by different mechanisms and consequently may be involved in different biological functions.

By studying both endogenous and stably, ectopically expressed HDAC4 in C2C12 cells, we have obtained evidence that subcellular localization of HDAC4 is uniquely and dynamically regulated during muscle differentiation. The analysis of HDAC4 and HDAC5 subcellular localization during C2C12 myoblast differentiation yielded another surprise in which we observed HDAC4 and HDAC5 to be present in nuclei of the myotubes (Fig. 1, G and L). This observation is consistent with the observation that C2C12 cells overexpressing HDAC4 tend to form large myotubes, which often contract vigorously in contrast to control C2C12 cells (Fig. 1, K-M, and data not shown). We note that our result is not in agreement with that reported by Lu et al. (1) in which nuclear HDAC4 and HDAC5 were shown to suppress C2C12 differentiation. This discrepancy could be due to the levels of ectopically expressed HDAC4 and HDAC5. Because only a single copy of infected gene is stably integrated per cell by retrovirus-mediated gene transfer while typically multiple integration events are achieved by transfection, it is possible that HDAC4 or -5 is expressed at much higher levels in the transfection system used by Lu et al.

Extremely high levels of HDAC4 or -5 may dominantly inhibit C2C12 differentiation. In any case, the presence of nuclear HDAC4 and -5 in terminally differentiated muscle cells is not consistent with the simple model that nuclear HDAC4 or -5 suppresses myogenesis. It is possible that nuclear HDAC4 is required to suppress the expression of certain genes for terminal muscle differentiation to proceed normally. Alternatively, nuclear HDAC4 might catalyze the deacetylation of non-histone proteins important in regulating C2C12 differentiation (20). Further studies will be needed to address these intriguing possibilities, our observations suggest a complex mode of regulation of the subcellular localization of HDAC4, which might play an important role in muscle differentiation.

In summary, we have shown that differential subcellular localization of HDAC4 can be controlled by a series of specific phosphorylation events. Based on our results, we propose a model (Fig. 4F) in which HDAC4 is constitutively phosphorylated by an unknown kinase. This phosphorylation leads to 14-3-3 binding. The nuclear HDAC4-14-3-3 complex but not HDAC4 alone can then respond to additional phosphorylation events mediated by other kinases, such as CaMKIV, in response to the initiation of specific signaling cascades. The second phosphorylation event may allow the interaction of HDAC4-14-3-3 with the Crm-1 export machinery, resulting in its efficient export from the nucleus.

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Addendum—During the preparation of this manuscript, McKinsey et al. (21) published a study describing that an active CaMKI can induce HDAC5 nuclear export.

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5 T. A. Bolger and T.-P. Yao, unpublished observation.
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