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SMRT-mediated co-shuttling enables export of class IIa HDACs independent of their CaM kinase phosphorylation sites

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Abstract
The Class IIa histone deacetylases (HDAC)4 and HDAC5 play a role in neuronal survival and behavioral adaptation in the CNS. Phosphorylation at 2/3 N-terminal sites promote their nuclear export. We investigated whether non-canonical signaling routes to Class IIa HDAC export exist because of their association with the co-repressor Silencing Mediator Of Retinoic And Thyroid Hormone Receptors (SMRT). We found that, while HDAC5 and HDAC4 mutants lacking their N-terminal phosphorylation sites (HDAC4 MUT, HDAC5 MUT) are constitutively nuclear, co-expression with SMRT renders them exportable by signals that trigger SMRT export, such as synaptic activity, HDAC inhibition, and Brain Derived Neurotrophic Factor (BDNF) signaling. We found that SMRT’s repression domain 3 (RD3) is critical for co-shuttling of HDAC5 MUT, consistent with the role for this domain in Class IIa HDAC association. In the context of BDNF signaling, we found that HDAC5 WT, which was more cytoplasmic than HDAC5 MUT, accumulated in the nucleus after BDNF treatment. However, co-expression of SMRT blocked BDNF-induced HDAC5 WT import in a RD3-dependent manner. In effect, SMRT-mediated HDAC5 WT export was opposing the BDNF-induced HDAC5 nuclear accumulation observed in SMRT’s absence. Thus, SMRT’s presence may render Class IIa HDACs exportable by a wider range of signals than those which simply promote direct phosphorylation.

Keywords: calcium channels, histone deacetylase, neurodegeneration, neurotrophic factor.

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to certain stimuli, including EGF-induced MEK1 signaling or Akt activation, and dissociation from chromatin in response to IκB kinase (Hong and Privalsky 2000; Hermanson et al. 2002; Hoberg et al. 2006; Perissi et al. 2004, 2010). In neurons, activity-dependent Ca2+ transients trigger SMRT export via a combination of MEK1 and CaM kinase pathways (McKenzie et al. 2005; Soriano et al. 2011). Other stimuli are also known to promote SMRT export, such as inhibition of Class I HDAC activity, specifically HDAC3 (Soriano and Hardingham 2011).

Thus, while CaM kinase signaling is capable of promoting the nuclear export of both Class IIa HDACs and SMRT, certain signaling pathways are selective for one or the other co-repressor. Given that SMRT and Class IIa HDAC family members associate with each other this raises the possibility, hitherto untested, that SMRT may be able to co-shuttle Class IIa HDACs out of the nucleus independent of the classical phosphorylation site-dependent mechanism. By studying the movement of ‘non-exportable’ phospho-site mutants of HDAC4 and 5, we find that SMRT, acting via its RD3 domain, is able to co-shuttle HDAC4/5 out of the nucleus in response to SMRT-exporting stimuli. Relevance of this pathway is shown in the context of BDNF signaling. We show that BDNF-induced MEK1 signaling promotes HDAC5 import, but that in the presence of SMRT this import is canceled out because of the promotion of SMRT export by MEK1 signaling which acts to co-shuttle HDAC5 back out of the nucleus via SMRT’s RD3 domain.

**Methods**

**Neuronal cultures and stimulations**

Cortical neurons from E21 Sprague Dawley rats were cultured as described (Leveille et al. 2010), growth medium was comprised of Neurobasal A medium + B27 (Invitrogen, Paisley, UK), 1% rat serum, 1 mM glutamine. Experiments were performed after a culturing period of 9–10 days during which cortical neurons develop a rich network of processes, express functional NMDA- and AMPA/kainate-type glutamate receptors, and form synaptic contacts. Stimulations were performed after transferring neurons into defined medium lacking trophic support ‘TMo’ (Papadia et al. 2005): 10% minimal essential medium (Invitrogen), 90% Salt-Glucose-Glycine (SGG) medium (SGG: 114 mM NaCl, 0.219% NaHCO3, 5.292 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 10 mM HEPES, 1 mM Glycine, 30 mM Glucose, 0.5 mM sodium pyruvate, 0.1% Phenol Red; osmolarity 325 mosm/L, hereafter TMo). Stimulations were initiated approximately 48 h after transfection. Bursts of action potential firing were induced by treatment of neurons with 50 μM bicuculline, and burst frequency was enhanced by addition of 250 μM 4-amino pyridine (Hardingham et al. 2001; Baxter et al. 2011). Trichostatin-A (TSA, 1 μM) was added for 8 h, which induces substantial histone H3 and H4 acetylation (Soriano et al. 2009b). BDNF (25 ng/mL) was also added for 8 h. extracellular signal-regulated kinase (ERK)1/2 inhibitor PD98059 (50 μM) was added 30 min before the stimulations.

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Plasmids
SMRT-myc and green fluorescent protein (GFP)-SMRT'S full length were gifts from Martin Privalsky [UC Davis; (Hong and Privalsky 2000)]. The MEF2 luciferase reporter containing three binding sites for MEF2 transcription factors was a gift from Eric Olson (Lu et al. 2000a). Plasmids GFP-SMRT^D3 (SMRT^Δ1018-1522), GFP-SMRT^Δ56 (GFP-SMRT^Δ1523-1854), and RD3-myc have been described elsewhere (Soriano and Hardingham 2011). The HDAC5^WT-Flag-GFP expression vector has been described previously (Belfield et al. 2006) and a non-exportable version of it (HDAC5^MUT-Flag-GFP) was generated by using overlap extension PCR to mutate serines 259 and 498 of HDAC5 to alanine residues. For this study, both HDAC5^WT-Flag-GFP and HDAC5^MUT-Flag-GFP vectors were modified for use in this study by introducing a stop codon between the Flag and GFP ORFs by site-directed mutagenesis (primer: TGA TGA TGA TAA ATC). The resulting vectors are referred to as HDAC5^WT-Flag and HDAC5^MUT-Flag. HDAC4^WT-Flag (a gift from Tony Kouzarides) was described previously, as has HDAC4^MUT-Flag containing mutations to its 3 CaM kinase sites S246/467/632A as described previously (Miska et al. 2001).

Transfections and immunofluorescence
Neurons were transfected using Lipofectamine 2000 (Invitrogen) as described (Soriano et al. 2006) and stimulated 48 h after transfection. Immunofluorescence was performed as described (Soriano et al. 2008). Cells were fixed in 4% paraformaldehyde, 3% sucrose in phosphate-buffered saline for 20 min. Permeabilization was performed with 0.5% NP-40 in phosphate-buffered saline. Primary antibodies used were anti-flag to visualize HDAC4/5 localization (1 : 1000; Sigma, Gillingham, UK), anti-GFP antibody to visualize SMRT localization (1 : 700; Invitrogen). Antibody binding was detected using biotinylated secondary antibody/fluorescein isothiocyanate-labeled streptavidin, and nuclei were counter-stained with 4',6-diamidino-2-phenylindole. Non-saturating pictures were taken on a Leica AF6000 LX imaging system (Leica Microsystems, Wetzlar, Germany), with a DFC350 FX digital camera and the subcellular distribution scored. For each treatment, ca. 150–200 cells were analyzed within three to five independent experiments. Distribution was scored either as exclusively nuclear or as containing observable cytoplasmic localization (Al-Mubarak et al. 2009).

Luciferase reporter assay
Firefly luciferase-based MEF2 reporter vector (MRE-Luc) was transfected along with a renilla expression vector (pTK-RL), and also, HDAC5^WT-Flag or HDAC5^MUT-Flag. Neurons were stimulated (where appropriate) for 8 h with BDNF (25 ng/mL) 40 h after transfection. Luciferase assays were performed using the Dual Glo assay kit (Promega, Fitchburg, Wisconsin, USA) with Firefly luciferase-based reporter activity normalized to the Renilla control (pTK-RL plasmid) in all cases.

Results
SMRT can mediate nuclear export of "non-exportable" mutants of Class IIa HDACs
Synaptic activity controls the expression of many genes through regulating the activity or expression of DNA-binding transcription factors (West et al. 2001; Papadia et al. 2008; Soriano et al. 2009a). However, activity-dependent signal pathways also influence the activity and location of broad-specificity transcriptional coactivators and co-repressors (Chawla et al. 2003; McKenzie et al. 2005; Soriano et al. 2011). We previously showed that synaptic activity promotes the nuclear export of SMRT in neurons mediated by both CaM kinase signaling and MEK1 signaling (McKenzie et al. 2005). As discussed above, SMRT is known to co-localize with Class IIa HDACs HDAC4 and HDAC5 in several cell types including in neurons and this association is important for SMRT’s function as a co-repressor. The localization of Class IIa HDACs is subject to dynamic regulation by nuclear CaM kinase activation, which phosphorylates HDAC4 and HDAC5, triggering nuclear export (Haberland et al. 2009). However, given the association of HDAC4/5 with SMRT, we hypothesized that SMRT may have the capacity to promote Class IIa HDAC export independent of the canonical direct phosphorylation pathway, in response to signals that trigger SMRT export.

To investigate this, we first utilized a mutant of HDAC5 lacking its two CaM kinase phosphorylation sites, rendering it non-exportable via the classical pathway. All HDAC constructs used in this study contained C-terminal flag tag enabling expression to be detected by immunofluorescence with an anti-flag antibody. In agreement with previous studies, in resting neurons, wild-type HDAC5 was predominantly nuclear but also at significant levels in the cytoplasm (Fig. 1a). In contrast, HDAC5^MUT was found to be almost exclusively nuclear (Fig. 1a). As expected, HDAC5^MUT was not exported from the nucleus in response to strong synaptic activity, while synaptic activity promoted the efficient nuclear export of HDAC5 (Fig. 1a and b), consistent with previous studies (Chawla et al. 2003). We next investigated whether co-expression of SMRT could affect the signal-responsiveness of the localization of HDAC5^MUT. We previously showed that HDAC5 and SMRT co-localize in the nucleus, and that in response to synaptic activity HDAC5 is first exported from the nucleus, followed by SMRT (McKenzie et al. 2005). Consistent with this, we found that co-expression of SMRT had no effect on the activity-dependent export of wild-type HDAC5. We also found that co-expression of HDAC5^MUT with SMRT had little effect on the basal localization of HDAC5^MUT (Fig. 1a and b). Strikingly, however, in the presence of SMRT, HDAC5^MUT was exported in response to synaptic activity (Fig. 1a and b). We also looked at the influence of SMRT on the localization of a ‘non-exportable’ mutant of HDAC4 [HDAC4^MUT].
SMRT’s RD3 domain is important for co-shuttling of HDAC5

We decided to investigate this putative co-shuttling mechanism further, focussing on HDAC5. Association of SMRT with Class IIa HDACs relies, at least in part on an interaction with SMRT’s RD3 domain (Huang et al. 2000; Fischle et al. 2002). We therefore investigated whether deletion of this domain affected the ability of SMRT to promote activity-dependent export of HDAC5MUT. We used a mutant of SMRT we made where amino acids 1018–1523, encompassing the RD3 domain were deleted (Fig. 2a, hereafter SMRTΔRD3). We confirmed our recent findings that SMRTΔRD3 undergoes activity-dependent export as does full-length SMRT (SMRTFL) (Soriano et al. 2011) (Fig. 2d). However, co-expression of SMRTΔRD3 was far worse at promoting activity-dependent export of HDAC5MUT than SMRTFL (Fig. 2b and c). As SMRT’s RD3 domain when expressed on its own is not subject to activity-dependent export (Soriano et al. 2011), we reasoned that expression of it, by competing with full-length HDAC5MUT for HDAC5, may interfere with activity-dependent export of HDAC5MUT by SMRTFL and this was indeed observed (Fig. 2e). Collectively, these experiments support a role for the Class IIa HDAC-interacting RD3 domain in SMRT-mediated co-shuttling of Class IIa HDACs.

HDAC inhibition promotes SMRT-mediated co-shuttling of HDAC5

We recently showed that inhibition of Class I HDAC (specifically HDAC3) activity promoted SMRT export via a mechanism dependent on its RD4 domain (Soriano and Hardingham 2011), confirmed in Fig. 3a. In that study, we presented data that support a model whereby SMRT’s RD4 region can recruit factors capable of mediating nuclear export of SMRT, but whose function and/or recruitment is suppressed by HDAC3 activity (Soriano and Hardingham 2011). As the RD4 domain is not required for activity-dependent export of SMRT (Soriano et al. 2011), we can exploit this
to further illustrate SMRT-mediated export of HDAC5MUT. Inhibition of HDAC activity by TSA treatment had little effect on basal HDAC5MUT nuclear localization in isolation (Fig. 3b). However, when SMRT was co-expressed, TSA strongly promoted SMRT-dependent HDAC5MUT export. HDAC5MUT-Flag was co-expressed in neurons together with full-length green fluorescent protein (GFP)-SMRT (SMRTFL). SMRT lacking the RD3 domain (GFP-SMRTΔRD3) or globin as a control plasmid (CON, here and thereafter) and were stimulated for 8 h with Bic or left unstimulated and the cellular localization of the HDAC5MUT-Flag was analyzed.

BDNF has opposing effects on SMRT and HDAC5 localization

Activity-dependent Ca2+ signals are capable of triggering CaM kinase-dependent HDAC5 export independent of any SMRT co-shuttling mechanism (Chawla et al. 2003). We therefore wanted to know what type of signal the co-shuttling mechanism could be relevant for, that is, a signal that triggers SMRT export but not direct nuclear Ca2+/CaM kinase-dependent HDAC5 export. We decided to study signaling by the neurotrophin BDNF, as activation of the BDNF receptor TrkB strongly activates ERK1/2 signaling, but evokes modest Ca2+ mobilization compared with action potential bursting. We found that exposure of neurons to BDNF caused SMRT export in a substantial proportion of neurons (Fig. 4a and b), and that this was reversed when ERK1/2 activation was blocked by pre-incubation with the MEK1 inhibitor PD98059 (Fig. 4a). We next studied the influence of BDNF signaling on wild-type HDAC5 localization expressed in the absence of SMRT. Not only did we find that BDNF signaling failed to promote HDAC5 export, it actually triggered increased nuclear localization of HDAC5 in an ERK1/2-dependent manner (Fig. 4c).
These observations indicate that BDNF treatment could affect gene expression mediated by HDAC5-repressed transcription factors such as MEF2s (McKinsey et al. 2001). To investigate this, we co-expressed a MEF2-reporter (Lu et al. 2000a) with either HDAC5WT or HDAC5MUT and assayed reporter activity in the presence or absence of BDNF treatment. BDNF treatment repressed MEF2 reporter activity in the presence of HDAC5WT (Fig. 4d), consistent with it increasing nuclear localization of HDAC5. Indeed, the effect of BDNF is to repress MEF2 reporter activity to levels observed when the constitutively nuclear HDAC5MUT is expressed. Moreover, BDNF has no additional effect in the presence of HDAC5MUT, further suggesting that BDNF is exerting its effects on MEF2-mediated transcription in the presence of HDAC5WT by promoting HDAC5 nuclear import (Fig. 4d).

Given that BDNF signaling promotes HDAC5 nuclear accumulation but SMRT export, and HDAC5 and SMRT associate with each other, we wanted to determine whether the presence of SMRT has any effect on the movement of HDAC5 following BDNF treatment. We found that when SMRT was co-expressed with HDAC5, BDNF-induced nuclear import of HDAC5 was completely blocked (Fig. 4e and g). Moreover, this inhibition of HDAC5 import by SMRT was dependent on SMRT’s HDAC5-interaction domain (RD3); expression of SMRTΔRD3 had no effect on BDNF-induced HDAC5 import (Fig. 4f and g), despite still being subjected to BDNF-induced export (Fig. 4h). Collectively, these observations suggest that the direct effect of BDNF on the nuclear accumulation of HDAC5 is being canceled out by the indirect, SMRT-mediated export. Analysis of HDAC5MUT localization confirmed this: BDNF had no effect on the nuclear localization of HDAC5MUT on its own (Fig. 4i). However, in the presence of SMRT, BDNF treatment resulted in the export of HDAC5MUT in a significant number of neurons (Fig. 4i). Thus, the presence of SMRT directly influences the signal-dependent localization of Class IIa HDACs.

**Discussion**

Subcellular localization of Class IIa HDACs represents the major mechanism to regulate their function. Phosphorylation at two or three conserved Serine residues at the N-terminus leads to interaction with 14-3-3, nuclear export and derepression of the target genes. Here, we have shown that...
in neurons Class IIa HDACs can be exported from the nucleus in an independent manner to the classical phosphorylation mechanism, by co-shuttling with SMRT. As several signals are known to be able to influence the subcellular localization of SMRT and its close relative N-CoR, an implication of this work is that the presence of SMRT/N-CoR may render Class IIa HDACs responsive to those signals, particularly given that Class IIa HDACs can exist in SMRT/N-CoR-containing complexes in the nucleus. We have shown that BDNF-induced MEK1 signaling promotes SMRT export but has the reverse effect on HDAC5, promoting import. However, when SMRT is present BDNF-induced HDAC5 import is blocked – likely because of direct import being canceled out by SMRT-mediated export. The influence of SMRT is lost upon deletion of its HDAC4/5-interacting RD3 domain, consistent
**Fig. 4** The presence of silencing mediator of retinoic and thyroid hormone receptors (SMRT) determines histone deacetylase (HDAC)5 cellular localization after brain derived neurotrophic factor (BDNF) stimulation. (a) BDNF induces SMRT nuclear export. Neurons were transfected with green fluorescent protein (GFP)-SMRT\(^{WT}\) and its cellular localization was analyzed after stimulation with BDNF (25 ng/mL) for 8 h. \(p < 0.05\) (n = 4). (b) Examples of the cellular localization of SMRT after treatment with BDNF. (c) BDNF promotes HDAC5 nuclear localization in an extracellular signal-regulated kinase (ERK)1/2-dependent manner. Neurons were transfected with HDAC5\(^{WT}\)-flag and its cellular localization was analyzed after stimulation with BDNF (25 ng/mL) for 8 h in absence or presence of the ERK1/2 inhibitor PD98059 (50 \(\mu M\)). \(p < 0.05\) (n = 6). (d) BDNF represses myocyte enhancer factor 2 (MEF2)-mediated gene expression. \(p < 0.05\) (n = 3). Neurons were transfected with a luciferase reporter containing 3 MEF2 binding sites plus either HDAC5\(^{WT}\)-flag or HDAC5\(^{WT}\)-flag, then treated with BDNF for 8 h, 40 h post-transfection. \(p < 0.05\) (n = 3). (e) BDNF-dependent HDAC5 nuclear import is blocked by SMRT. Neurons were transfected with HDAC5\(^{WT}\)-flag in the presence or absence of GFP-SMRT and HDAC5\(^{WT}\)-flag cellular localization was analyzed after stimulation with BDNF (25 ng/mL) for 8 h. \(p < 0.05\) (n = 5). (f) Inhibition of BDNF mediated HDAC5 export is dependent of SMRT’s repression domain 3 (RD3) domain. HDAC5\(^{WT}\)-flag was co-expressed in neurons together with full-length GFP-SMRT lacking the RD3 domain (SMRT\(^{ΔRD3}\)) or globin as a control plasmid (CON) and 48 h after transfection the neurons were stimulated for 8 h with BDNF or left unstimulated and the cellular localization of the HDAC5\(^{WT}\)-flag was analyzed. \(p < 0.05\) (n = 4). (g) Representative examples of the cellular localization of HDAC5\(^{WT}\)-flag induced by BDNF in absence or presence of the indicated GFP-SMRT constructs. (h) GFP-SMRT\(^{ΔRD3}\) is exported by BDNF. Analysis of GFP-SMRT\(^{ΔRD3}\) cellular localization after BDNF treatment. \(p < 0.05\) (n = 4). (i) BDNF mediated SMRT export promotes HDAC5\(^{MUT}\) export. Neurons were transfected with HDAC5\(^{MUT}\)-flag in the presence or absence of GFP-SMRT and HDAC5\(^{MUT}\)-flag cellular localization was analyzed after stimulation with BDNF (25 ng/mL) for 8 h. \(p < 0.05\) (n = 4).

To conclude, the prominent role that Class IIa HDACs play in controlling neuronal survival and death, and differentiation, as well as certain behavioral responses, such as addiction, make an understanding of signals that control their subcellular localization to be of direct importance. Outside of the CNS, the roles played by Class IIa HDACs in regulating transcriptional programs relating to skeletal myogenesis, cardiac hypertrophy, and thymocyte development (Lu et al. 2000b; McKinsey et al. 2000; Zhang et al. 2002; Haberland et al. 2009; Parra and Verdin 2010; Watson et al. 2012) raise the question as to whether these functions may be in influenced by signals that promote SMRT-mediated co-shuttling independent of the classical export pathways.

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