



**In Cortical Neurons, Silencing Mediator Of Retinoic And Thyroid Hormone Receptors (SMRT)-Mediated Co-Shuttling Enables Export Of Class IIa Histone Deacetylases Independent Of The Classical CaM Kinase Phosphorylation Site-Dependent Mechanism**

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**In Cortical Neurons, Silencing Mediator Of Retinoic And Thyroid Hormone Receptors  
(SMRT)-Mediated Co-Shuttling Enables Export Of Class IIa Histone Deacetylases  
Independent Of The Classical CaM Kinase Phosphorylation Site-Dependent  
Mechanism**

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**Running title:** *SMRT-mediated export of Class IIa HDACs*

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**Keywords:** Histone deacetylase, Calcium channels, Neurodegeneration, Neurotrophic factor

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**Abbreviations:** SMRT, silencing mediator of retinoic and thyroid hormone receptors; RD3,  
repression domain 3; HDAC, histone deacetylase; BDNF, brain derived neurotrophic factor;

*SMRT-mediated export of Class IIa HDACs*

HAT, histone acetyltransferase; CaM kinases, Ca<sup>2+</sup>/calmodulin-dependent protein kinases;;

GFP, green fluorescent protein; 4-AP, 4-amino pyridine.

For Peer Review

**ABSTRACT**

The Class IIa histone deacetylases HDAC4 and HDAC5 play a role in neuronal survival and behavioural 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 due to 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<sup>MUT</sup>, HDAC5<sup>MUT</sup>) 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<sup>MUT</sup>, consistent with the role for this domain in Class IIa HDAC association. In the context of BDNF signalling, we found that HDAC5<sup>WT</sup>, which was more cytoplasmic than HDAC5<sup>MUT</sup>, accumulated in the nucleus after BDNF treatment. However, co-expression of SMRT blocked BDNF-induced HDAC5 import in a RD3-dependent manner. In effect, SMRT-mediated HDAC5 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.

## INTRODUCTION

Histone acetylation and deacetylation plays an important regulatory role in gene transcription, with acetylation strongly associated with increased transcriptional activity (for review (Haberland *et al.* 2009, Perissi *et al.* , Perissi *et al.* 2010)). Acetylation/deacetylation of histones is a dynamic process that depends on the balance between activity of histone acetyltransferases (HAT) and histone deacetylases (HDAC). The HDACs are a large protein family named after their ability to remove acetyl groups from lysine residues located within the N-terminal tail of histones ((Haberland *et al.* 2009, Perissi *et al.* 2010)) but can act on non-histones as well. In mammals there are five classes of HDACs (Class I, IIa, IIb, III and IV) containing many different HDACs (18 in humans). Class IIa HDACs (HDAC4, 5, 7, 9), unlike other HDACs, are expressed in a tissue-specific manner with high expression levels in skeletal, cardiac, and smooth muscle, bone, the immune system, the vascular system, and the brain (reviewed in (Parra & Verdin 2010)). Studies with knockout mice for various Class IIa HDACs show their important function in differentiation and developmental processes such as formation of skeletal muscle, cardiac hypertrophy, bone development and T-cell differentiation (Haberland *et al.* 2009, Parra & Verdin 2010, Martin *et al.* 2007). In the CNS Class IIa HDACs controls neuronal survival ((Linseman *et al.* 2003, Bolger & Yao 2005, Chen & Cepko 2009), differentiation (Schneider *et al.* 2008), long-term-memory-related synaptic plasticity (Guan *et al.* 2002) and behavioural responses ((Tsankova *et al.* 2006, Renthall *et al.* 2007, Taniguchi *et al.* 2011).

A characteristic of Class IIa HDACs is that their ability to suppress transcription in the nucleus is subject to dynamic regulation. Class IIa HDACs are subject to phosphorylation on two or three conserved serine residues in their N-termini domain and this modification exposes a nuclear export signal and creates a binding site for 14-3-3 proteins in the cytoplasm

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3 which anchor them there, thus preventing them from acting as transcriptional corepressors  
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5 (McKinsey *et al.* 2001, Martin *et al.* 2007, Parra & Verdin 2010). Mutation of these  
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7 phosphorylation sites renders Class IIa HDACs "non-exportable". The primary kinase family  
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9 responsible for signal-dependent Class IIa phosphorylation and subsequent export are the  
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11  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinases (CaM kinases), although other kinases such as  
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13 PKD and SIK are also capable of acting at these sites. In neurons, the major Class IIa HDACs  
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15 expressed are HDAC4 and HDAC5, and these are subject to activity- and CaM kinase-  
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17 dependent nucleo-cytoplasmic shuttling (Chawla *et al.* 2003). Moreover, CaM kinase-  
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19 dependent nuclear exclusion of HDAC 5 has been implicated in promoting the survival of  
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21 neurons (Linseman *et al.* 2003). HDAC4, in contrast may play pro-survival or pro-death roles  
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23 in the nucleus which may depend on the neuron's developmental stage (Bolger & Yao 2005,  
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25 Majdzadeh *et al.* 2008). HDAC4 has been additionally shown to have an *active* pro-survival  
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27 role in retinal neurons when resident in the cytoplasm (Chen & Cepko 2009). Furthermore  
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29 alterations to Class IIa activity mediate the actions of certain anti-depressants and control  
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31 addiction pathways (Taniguchi *et al.* 2011, Tsankova *et al.* 2006, Renthal *et al.* 2007). Thus,  
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33 signaling mechanisms that alter the subcellular localization of Class IIa HDACs have the  
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35 capacity to influence important aspects of neuronal fate and function.  
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41 Class IIa HDACs can exist in the nucleus as part of multi-protein corepressor  
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43 complexes centred on SMRT or its close relative N-CoR, and containing other corepressor  
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45 proteins and Class I HDACs, particularly HDAC3 (Guenther *et al.* 2001, Huang *et al.* 2000,  
46  
47 Haberland *et al.* 2009, Perissi *et al.* 2004, Watson *et al.* 2012). Indeed, one function of Class  
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49 IIa HDACs is thought to be as a physical bridge between the SMRT/N-CoR complex and  
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51 certain transcription factors such as MEF2, rather than contributing substantial HDAC  
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53 activity as such (Fischle *et al.* 2002, Lahm *et al.* 2007). Interaction of Class IIa HDACs with  
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55 SMRT is through SMRT's repression domain 3 (RD3; (Huang *et al.* 2000, Fischle *et al.* 2002).  
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*SMRT-mediated export of Class IIa HDACs*

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3 SMRT or N-CoR are known to undergo nuclear export in response to certain stimuli,  
4 including EGF-induced MEK1 signaling or Akt activation, and dissociation from chromatin  
5 in response to I $\kappa$ B kinase (Hong & Privalsky 2000, Hoberg *et al.* 2006, Perissi *et al.* 2010,  
6 Hermanson *et al.* 2002). In neurons, activity-dependent Ca<sup>2+</sup> transients trigger SMRT export  
7 via a combination of MEK1 and CaM kinase pathways (McKenzie *et al.* 2005, Soriano *et al.*  
8 2011). Other stimuli are also known to promote SMRT export, such as inhibition of Class I  
9 HDAC activity, specifically HDAC3 (Soriano & Hardingham 2011).  
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20 Thus, while CaM kinase signaling is capable of promoting the export of both Class IIa  
21 HDACs and SMRT, certain signaling pathways are selective for one or the other corepressor.  
22 Given that SMRT and Class IIa HDAC family members associate with each other this raises  
23 the possibility, hitherto untested, that SMRT may be able to co-shuttle Class IIa HDACs out  
24 of the nucleus independent of the classical phosphorylation site-dependent mechanism. By  
25 studying the movement of "non exportable" phospho-site mutants of HDAC4 and 5, we find  
26 that SMRT, acting via its RD3 domain, is able to co-shuttle HDAC4/5 out of the nucleus in  
27 response to SMRT-exporting stimuli. Relevance of this pathway is shown in the context of  
28 BDNF signalling. We show that BDNF-induced MEK1 signaling promotes HDAC5 import,  
29 but that in the presence of SMRT this import is cancelled out due to the promotion of SMRT  
30 export by MEK1 signalling which acts to co-shuttle HDAC5 back out of the nucleus via  
31 SMRT's RD3 domain.  
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## EXPERIMENTAL PROCEDURES

*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), 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-type 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% MEM (Invitrogen), 90% Salt-Glucose-Glycine (SGG) medium (SGG: 114 mM NaCl, 0.219 % NaHCO<sub>3</sub>, 5.292 mM KCl, 1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 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). TSA (1 μM) or BDNF (25 ng/ml) were added for 8 hours. ERK1/2 inhibitor PD98059 (50 μM) was added 30 minutes before the stimulations at.

*Plasmids*-SMRT-myc and GFP-SMRT $\alpha$  full length were gifts from Martin Privalsky (UC Davis; (Hong & Privalsky 2000)). Plasmids GFP-SMRT<sup>RD3</sup> (SMRT $\Delta$ (1018-1523)), GFP-SMRT<sup>ARD4</sup> (GFP-SMRT $\Delta$ (1523-1854)) and RD3-myc have been described elsewhere (Soriano & Hardingham 2011). The HDAC5-Flag-GFP expression vector has been described previously (Belfield *et al.* 2006) and a non-exportable version of it (HDAC5<sup>MUT</sup>-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-Flag-GFP and HDAC5<sup>MUT</sup>-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 T<sub>t</sub>G A<sub>t</sub>a GGT GAG



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3 CAA GGG CG, underlined nucleotides indicate mutations) to prevent expression of the GFP  
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5 portion. The resulting vectors are referred to as HDAC5-Flag and HDAC5<sup>MUT</sup>-Flag. HDAC4-  
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7 Flag has been described previously as has HDAC4<sup>MUT</sup>-Flag containing mutations to its 3  
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9 CaM kinase sites S246/467/632A as described previously (Miska *et al.* 2001).  
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11 *Transfections and immunofluorescence-* Neurons were transfected using  
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13 Lipofectamine 2000 (Invitrogen) and stimulated 48 hr after transfection. Immunofluorescence  
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15 was performed as described (Soriano *et al.* 2008). Cells were fixed in 4% paraformaldehyde,  
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17 3% sucrose in PBS for 20 mins. Permeabilization was performed with 0.5% NP-40 in PBS.  
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19 Primary antibodies used were anti-flag to visualise HDAC4/5 localisation (1:1000; Sigma),  
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21 anti-GFP antibody to visualise SMRT localisation (1:700; Invitrogen). Antibody binding was  
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23 detected using biotinylated secondary antibody/cy3-conjugated streptavidin, and nuclei were  
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25 counter-stained with DAPI. Non-saturating pictures were taken on a Leica AF6000 LX  
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27 imaging system, with a DFC350 FX digital camera and the subcellular distribution scored.  
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29 For each treatment, ca. 150-200 cells were analysed within 3-5 independent experiments.  
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31 Distribution was scored either as exclusively nuclear or as containing observable cytoplasmic  
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33 localization.  
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38 *Statistical analysis-*Statistical testing involved a 2-tailed paired student T-test. For any  
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40 multiple comparisons within data sets we used a one-way ANOVA followed by Fisher's LSD  
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42 post-hoc test.  
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## RESULTS

*SMRT can mediate export of "non-exportable" mutants of Class IIa HDACs*-We previously showed that synaptic activity promotes the export of SMRT in neurons mediated by both CaM kinase signalling and MEK1 signaling (McKenzie et al. 2005). As discussed above, SMRT is known to colocalize 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 corepressor. The localization of Class IIa HDACs are 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<sup>MUT</sup> was found to be almost exclusively nuclear (Fig. 1a). As expected, HDAC5<sup>MUT</sup> was not exported in response to strong synaptic activity, while, synaptic activity promoted the efficient nuclear export of HDAC5 (Fig. 1a,b), consistent with previous studies (Chawla et al. 2003). We next investigated whether co-expression of SMRT could effect the signal-responsiveness of the localization of HDAC5<sup>MUT</sup>. We previously showed that HDAC5 and SMRT colocalize in the nucleus, and that in response to synaptic activity HDAC5 is first exported from the nucleus, followed by SMRT (McKenzie

*SMRT-mediated export of Class IIa HDACs*

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3 et al. 2005). Consistent with this, we found that co-expression of SMRT had no effect on the  
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5 activity-dependent export of wild-type HDAC5. We also found that co-expression of  
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7 HDAC5<sup>MUT</sup> with SMRT had little effect on the basal localization of HDAC5<sup>MUT</sup> (Fig. 1a,b).  
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9 Strikingly however, in the presence of SMRT, HDAC5<sup>MUT</sup> was exported in response to  
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11 synaptic activity (Fig. 1a,b). We also looked at the influence of SMRT on the localization of a  
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13 'non-exportable' mutant of HDAC4 (HDAC4<sup>MUT</sup>, (Grozinger & Schreiber 2000)) containing  
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15 serine-to-alanine mutations at serines 246/467/632. Wild-type HDAC4 was not studied  
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17 because in neurons under our experimental conditions it was constitutively cytoplasmic (data  
18  
19 not shown), in agreement with previous studies (Chawla et al. 2003). We found that co-  
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21 expression of SMRT resulted in HDAC4<sup>MUT</sup> being exported in response to synaptic activity  
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23 (Fig. 1a). Thus, the 'non-exportable' mutants of HDAC4 and HDAC5 can be rendered  
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25 exportable by SMRT co-expression, suggestive of a non-canonical pathway to Class IIa  
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27 HDAC export.  
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32 *SMRT's RD3 domain is important for co-shuttling of HDAC5*—We decided to  
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34 investigate this putative co-shuttling mechanism further, focussing on HDAC5. Association  
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36 of SMRT with Class IIa HDACs relies, at least in part on an interaction with SMRT's RD3  
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38 domain (Fischle et al. 2002, Huang et al. 2000). We therefore investigated whether deletion of  
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40 this domain affected the ability of SMRT to promote activity-dependent export of  
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42 HDAC5<sup>MUT</sup>. We used a mutant of SMRT we made where amino acids 1018-1523,  
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44 encompassing the RD3 domain were deleted (Fig. 2a, hereafter SMRT<sup>ΔRD3</sup>). We confirmed  
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46 our recent findings that SMRT<sup>ΔRD3</sup> undergoes activity dependent export as does full length  
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48 SMRT (SMRT<sup>FL</sup>) (Soriano et al. 2011) (Fig. 2d). However, co-expression of SMRT<sup>ΔRD3</sup> was  
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50 far worse at promoting activity-dependent export of HDAC5<sup>MUT</sup> than SMRT<sup>FL</sup> (Fig. 2b,c).  
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52 Since SMRT's RD3 domain when expressed on it's own is not subject to activity dependent  
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54 export (Soriano et al. 2011), we reasoned that expression of it, by competing with full-length  
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3 HDAC5<sup>MUT</sup> for HDAC5, may interfere with activity-dependent export of HDAC5<sup>MUT</sup> by  
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5 SMRT<sup>FL</sup> and this was indeed observed (Fig. 2e). Collectively, these experiments support a  
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7 role for the Class IIa HDAC-interacting RD3 domain in SMRT-mediated co-shuttling of  
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9 Class IIa HDACs.  
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11 *HDAC inhibition promotes SMRT-mediated co-shuttling of HDAC5*—We recently  
12 showed that inhibition of Class I HDAC (specifically HDAC3) activity promoted SMRT  
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14 export via a mechanism dependent on its RD4 domain (Soriano & Hardingham 2011),  
15  
16 confirmed in Fig. 3a. Since the RD4 domain is not required for *activity*-dependent export of  
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18 SMRT (Soriano et al. 2011), we can exploit this to further illustrate SMRT-mediated export  
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20 of HDAC5<sup>MUT</sup>. Inhibition of HDAC activity by TSA treatment had little effect on basal  
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22 HDAC5<sup>MUT</sup> nuclear localization in isolation (Fig. 3b). However, when SMRT was co-  
23  
24 expressed, TSA strongly promoted SMRT export in a manner dependent on the RD4 domain  
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26 (Fig. 3b). By comparison, activity-dependent SMRT export is independent of the RD4  
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28 domain (Soriano & Hardingham 2011), Fig. 3c and consistent with that, SMRT-mediated  
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30 HDAC5<sup>MUT</sup> export in response to synaptic activity was independent of the RD4 domain (Fig.  
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32 3d).  
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38 *BDNF has opposing effects on SMRT and HDAC5 localization*—Activity-dependent  
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40 Ca<sup>2+</sup> signals are capable of triggering CaM kinase-dependent HDAC5 export independent of  
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42 any SMRT co-shuttling mechanism (Chawla et al. 2003). We therefore wanted to know what  
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44 type of signal the co-shuttling mechanism could be relevant for i.e. a signal that triggers  
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46 SMRT export but not direct nuclear Ca<sup>2+</sup>/CaM kinase-dependent HDAC5 export. We decided  
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48 to study signaling by the neurotrophin BDNF, since activation of the BDNF receptor TrkB  
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50 strongly activates ERK1/2 signaling, but evokes modest Ca<sup>2+</sup> mobilization compared to action  
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52 potential bursting. We found that exposure of neurons to BDNF caused SMRT export in a  
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54 substantial proportion of neurons (Fig. 4a,b), and that this was reversed when ERK1/2  
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*SMRT-mediated export of Class IIa HDACs*

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3 activation was blocked by preincubation with the MEK1 inhibitor PD98059 (Fig. 4a). We  
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5 next studied the influence of BDNF signaling on wild-type HDAC5 localization expressed in  
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7 the absence of SMRT. Not only did we find that BDNF signaling failed to promote HDAC5  
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9 export, it actually triggered increased nuclear localization of HDAC5 in a ERK1/2-dependent  
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11 manner (Fig. 4c,f).  
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14 Given that BDNF-induced ERK1/2 activity promotes HDAC5 nuclear accumulation  
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16 but SMRT export, and HDAC5 and SMRT associate with each other, we wanted to determine  
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18 whether the presence of SMRT has any effect on the movement of HDAC5 following BDNF  
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20 treatment. We found that when SMRT was co-expressed with HDAC5, BDNF-induced  
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22 import of HDAC5 was completely blocked (Fig. 4d,f). Moreover, this inhibition of HDAC5  
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24 import by SMRT was dependent on SMRT's HDAC5-interaction domain (RD3): expression  
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26 of SMRT<sup>ARD3</sup> had no effect on BDNF-induced HDAC5 import (Fig. 4e,f), despite still being  
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28 subjected to BDNF-induced export (Fig. 4g). Collectively these observations suggest that the  
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30 direct effect of BDNF on the nuclear accumulation of HDAC5 is being cancelled out by the  
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32 indirect, SMRT-mediated export. Analysis of HDAC5<sup>MUT</sup> localization confirmed this: BDNF  
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34 had no effect on the nuclear localization of HDAC5<sup>MUT</sup> on its own (Fig. 4h). However, in the  
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36 presence of SMRT, BDNF treatment resulted in the export of HDAC5<sup>MUT</sup> in a significant  
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38 number of neurons (Fig. 4h). Thus, the presence of SMRT directly influences the signal-  
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40 dependent localization of Class IIa HDACs.  
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## 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. Since 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 due to direct import being cancelled out by SMRT-mediated export. The influence of SMRT is lost upon deletion of its HDAC4/5-interacting RD3 domain, consistent with a direct co-shuttling mechanism. Other signals may also impact on Class IIa HDAC localisation in the presence of SMRT/N-CoR, such as the PI3-kinase/Akt pathway, which in response to CNTF treatment mediates the nuclear export of N-CoR in neural stem cells (Hermanson et al. 2002).

Although this is the first time that Class IIa HDAC export has been demonstrated totally independent of their signal-responsive phosphorylation sites, co-shuttling of one class member HDAC5, by another (HDAC4) has been shown (Backs *et al.* 2008). Unlike CaM kinases IV and I, which can promote export of both HDAC4 and 5, CaM kinase II can only promote HDAC4 export because HDAC5 lacks a CaM kinase II docking site (Backs *et al.* 2006). However, in the presence of HDAC4, HDAC5 can be co-shuttled out of the nucleus by

*SMRT-mediated export of Class IIa HDACs*

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3 CaM kinase II by interacting with HDAC4. Even "non-exportable" HDAC5<sup>MUT</sup> can be co-  
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5 shuttled in this way, suggestive of "piggy-pack" export. However, a certain degree of export  
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7 is also observed if "non-exportable" HDAC4<sup>MUT</sup> is paired with wild-type HDAC5, indicating  
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9 that docking of CaM kinase II on HDAC4 followed by trans-phosphorylation of HDAC5 can  
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11 also lead to export (Backs et al. 2006). Importantly, when HDAC5<sup>MUT</sup> and HDAC4<sup>MUT</sup> are  
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13 co-expressed there is no export in response to CaM kinase II activity: phosphorylation sites  
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15 on either HDAC4 or HDAC5 are essential for the export to take place, unlike the situation we  
16  
17 describe where export of HDAC5<sup>MUT</sup> or HDAC4<sup>MUT</sup> can be promoted by SMRT. Of note,  
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19 Class IIa HDACs can themselves co-shuttle other proteins in response to CaM kinase  
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21 signalling, through direct interaction. For example, both HDAC4 and HDAC5 physically  
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23 associate with ankyrin-repeat proteins ANKRA2 and RFXANK. Phosphorylation of HDAC  
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25 by CaM kinases promotes the nuclear export of the HDAC/ANKRA2 and HDAC/RFXANK  
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27 complexes but not if the HDAC used is a non-exportable mutant (McKinsey *et al.* 2006).  
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32 To conclude, the prominent role that Class IIa HDACs play in controlling neuronal  
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34 survival and death, and differentiation, as well as certain behavioural responses such as  
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36 addiction, make an understanding of signals that control their subcellular localization to be of  
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38 direct importance. Outside of the CNS, the roles played by Class IIa HDACs in regulating  
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40 transcriptional programs relating to skeletal myogenesis, cardiac hypertrophy and thymocyte  
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42 development (McKinsey *et al.* 2000, Lu *et al.* 2000, Zhang *et al.* 2002, Parra & Verdin 2010,  
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44 Haberland *et al.* 2009, Watson *et al.* 2012) raise the question as to whether these functions  
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46 may be influenced by signals that promote SMRT-mediated co-shuttling independent of the  
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48 classical export pathways.  
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For Peer Review



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**FIGURE LEGENDS****FIGURE 1. SMRT mediates non-canonical pathway to Class IIa HDAC nuclear export.**

A) Class IIa HDACs lacking CaMKIV phosphorylation sites (HDAC<sup>MUT</sup>) can be exported when co-expressed with SMRT. Neurons were transfected with the indicated HDAC-flag constructs in the presence or absence of full length GFP-SMRT and 48 h after transfection the neurons were stimulated for 8 h with bicuculline plus 4-AP (labelled BiC in this and subsequent figures) or left unstimulated and the cellular localization of the HDAC-flags was analyzed. \*p < 0.05 (n=3-5). B) Examples of the cellular localization of the indicated flag-tagged HDAC5s in absence or presence of SMRT after treatment with BiC for 8 h. Scale bar is 20  $\mu$ m here and thereafter.

**FIGURE 2. SMRT's RD3 domain is important for mediating export of HDAC5. A)**

Schematic of SMRT deletion mutants/fragments used in this study. The red box indicates a myc tag. B) Deletion of SMRT RD3 interacting domain impairs SMRT dependent HDAC5<sup>MUT</sup> export. HDAC5<sup>MUT</sup>-Flag was co-expressed in neurons together with full length GFP-SMRT (SMRT<sup>FL</sup>), SMRT lacking the RD3 domain (GFP-SMRT <sup>$\Delta$ RD3</sup>) 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 HDAC5<sup>MUT</sup>-Flag was analyzed. \*p < 0.05 (n=3). C) Examples of the cellular localization of HDAC5<sup>MUT</sup>-flag when co-expressed with the indicated GFP-SMRT variants after treatment with BiC for 8 h. D) GFP-SMRT<sup>FL</sup> and SMRT <sup>$\Delta$ RD3</sup> are exported by synaptic activity. Analysis of cellular localization of SMRT<sup>FL</sup> or GFP-SMRT <sup>$\Delta$ RD3</sup> after stimulation with BiC for 8 h. \*p < 0.05 (n=3). E) Disruption of HDAC5 interaction with SMRT impairs SMRT dependent HDAC5<sup>MUT</sup> export. Neurons were co-

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transfected with HDAC5<sup>MUT</sup>-flag plus GFP-SMRT<sup>FL</sup> and the SMRT's RD3-myc domain to disrupt SMRT-HDAC interaction or globin (control) and HDAC5<sup>MUT</sup>-flag cellular localization was analyzed after stimulation with BiC. \*p < 0.05 (n=3).

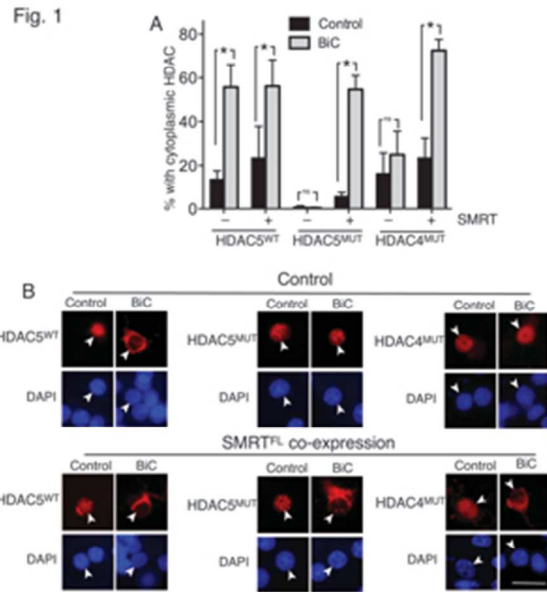
**FIGURE 3. SMRT dependent HDAC<sup>MUT</sup> export requires SMRT export.** A) TSA dependent SMRT export relies on SMRT's RD4 domain. Neurons were transfected with GFP-SMRT<sup>FL</sup> or GFP-SMRT lacking the RD4 domain (SMRT<sup>ΔRD4</sup>) and stimulated with TSA (1 μM) for 8 h and cellular the localization was analyzed. \*p < 0.05 (n=4). B) Deletion of SMRT RD4 interacting domain abolish TSA induced SMRT dependent HDAC5<sup>MUT</sup> export. HDAC5<sup>MUT</sup>-Flag was co-expressed with the indicated SMRT constructs, stimulated for 8 h with TSA and the cellular localization of the flag-HDAC5<sup>MUT</sup> was analyzed. \*p < 0.05 (n=4-5). C) Synaptic activity dependent SMRT export does not require the RD4 domain. Neurons were transfected with GFP-SMRT<sup>FL</sup> or GFP-SMRT<sup>ΔRD4</sup> and 48 h later stimulated with BiC for 8 h and cellular the localization was analyzed. \*p < 0.05 (n=5). D) Deletion of SMRT RD4 interacting does not affect the synaptic activity induced SMRT dependent HDAC5<sup>MUT</sup> export. HDAC5<sup>MUT</sup>-flag was co-expressed with the indicated SMRT constructs, stimulated for 8 h with BiC and the cellular localization of the HDAC5<sup>MUT</sup>-Flag was analyzed. \*p < 0.05 (n=4-5).

**FIGURE 4. The presence of SMRT determines HDAC5 cellular localization after BDNF stimulation.** A) BDNF induces SMRT nuclear export. Neurons were transfected with GFP-SMRT<sup>FL</sup> and its cellular localization was analyzed after stimulation with BDNF (25 ng/ml) for 8h. \*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 ERK1/2 dependent manner. Neurons were transfected with HDAC5<sup>WT</sup>-flag and its cellular localization was

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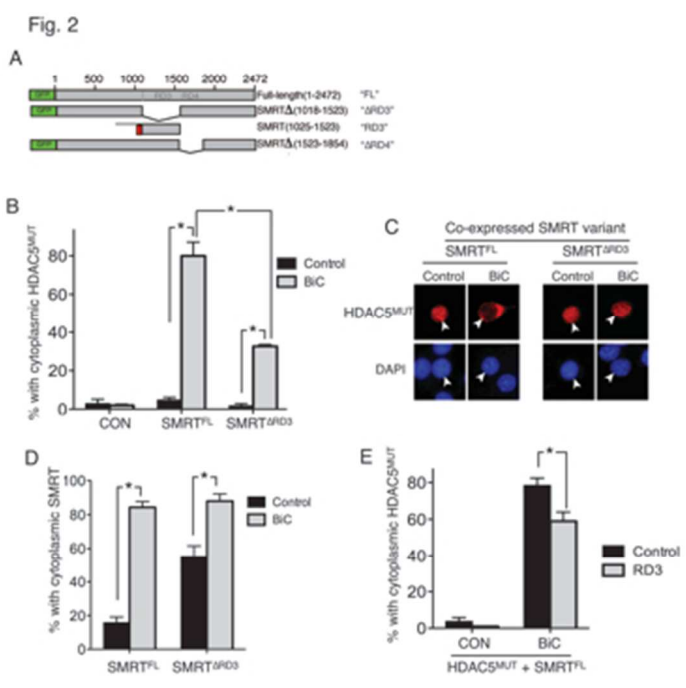
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3 analyzed after stimulation with BDNF (25 ng/ml) for 8h in absence or presence of the  
4 ERK1/2 inhibitor PD98059 (50  $\mu$ M). \*p < 0.05 (n=6). D) BDNF dependent HDAC5 nuclear  
5 import is blocked by SMRT. Neurons were transfected with HDAC5<sup>WT</sup>-flag in the presence  
6 or absence of GFP-SMRT and HDAC5<sup>WT</sup>-flag cellular localization was analyzed after  
7 stimulation with BDNF (25 ng/ml) for 8h. \*p < 0.05 (n=5). E) Inhibition of BDNF mediated  
8 HDAC5 import is dependent of SMRT's RD3 domain. HDAC5<sup>WT</sup>-flag was co-expressed in  
9 neurons together with full length GFP-SMRT lacking the RD3 domain (SMRT <sup>$\Delta$ RD3</sup>) or globin  
10 as a control plasmid (CON) and 48 h after transfection the neurons were stimulated for 8 h  
11 with BDNF or left unstimulated and the cellular localization of the HDAC5<sup>WT</sup>-flag was  
12 analyzed. \*p < 0.05 (n=4). F) Representative examples of the cellular localization of  
13 HDAC5<sup>WT</sup>-flag induced by BDNF in absence or presence of the indicated GFP-SMRT  
14 constructs. G) GFP-SMRT <sup>$\Delta$ RD3</sup> is exported by BDNF. Analysis of GFP-SMRT <sup>$\Delta$ RD3</sup> cellular  
15 localization after BDNF treatment. \*p < 0.05 (n=4). H) BDNF mediated SMRT export  
16 promotes HDAC5<sup>MUT</sup> export. Neurons were transfected with HDAC5<sup>MUT</sup>-flag in the presence  
17 or absence of GFP-SMRT and HDAC5<sup>MUT</sup>-flag cellular localization was analyzed after  
18 stimulation with BDNF (25 ng/ml) for 8h. \*p < 0.05 (n=4).  
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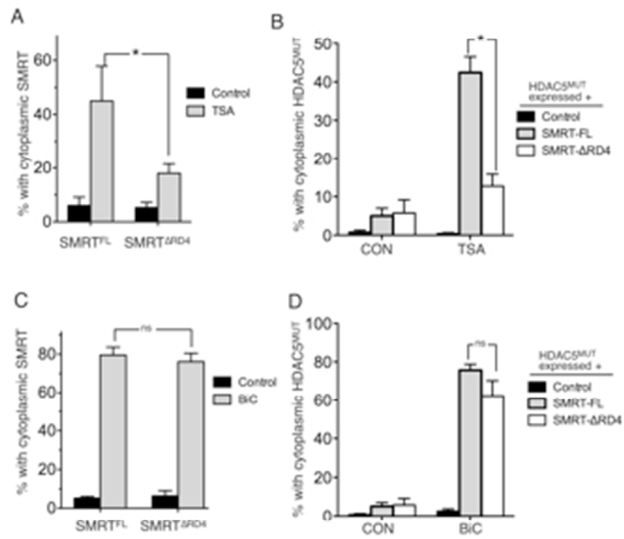
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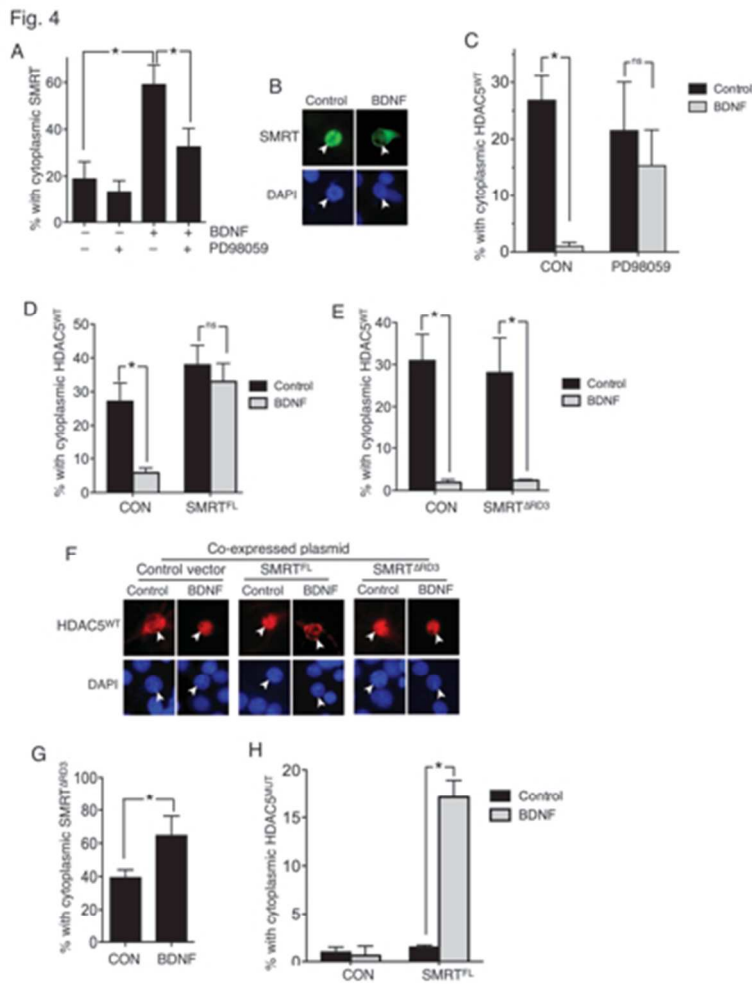


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Fig. 3



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