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HDAC inhibitors impair *Fshb* subunit expression in murine gonadotrope cells

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Abstract

Fertility is dependent on follicle-stimulating hormone (FSH), a product of gonadotrope cells of the anterior pituitary gland. Hypothalamic gonadotropin-releasing hormone (GnRH) and intra-pituitary activins are regarded as the primary drivers of FSH synthesis and secretion. Both stimulate expression of the FSH beta subunit gene (*Fshb*), although the underlying mechanisms of GnRH action are poorly described relative to those of the activins. There is currently no consensus on how GnRH regulates *Fshb* transcription, as results vary across species and between *in vivo* and *in vitro* approaches. One of the more fully developed models suggests that the murine *Fshb* promoter is tonically repressed by histone deacetylases (HDACs) and that GnRH relieves this repression, at least in immortalized murine gonadotrope-like cells (LβT2 and αT3-1). In contrast, we observed that the class I/II HDAC inhibitor trichostatin A (TSA) robustly inhibited basal, activin A-, and GnRH-induced *Fshb* mRNA expression in LβT2 cells and in primary murine pituitary cultures. Similar results were obtained with the class I specific HDAC inhibitor, entinostat, whereas two class II-specific inhibitors, MC1568 and TMP269, had no effects on *Fshb* expression. Collectively, these data suggest that class I HDACs are positive, not negative, regulators of *Fshb* expression *in vitro* and that, contrary to earlier reports, GnRH may not stimulate *Fshb* by inhibiting HDAC-mediated repression of the gene.

Key Words

- FSH
- histone deacetylase
- activin
- GnRH
- gonadotropes

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Introduction

Follicle-stimulating hormone (FSH) is an essential regulator of mammalian fertility (Kumar *et al.* 1997, Tapanainen *et al.* 1997). FSH is composed of two subunits: chorionic gonadotropin alpha (CGA, encoded by the *Cga* gene), which is shared with the other members of the glycoprotein hormone family, and a hormone-specific beta subunit (FSHB, encoded by the *Fshb* gene), which confers receptor-binding specificity. According to both human and rodent studies, loss-of-function mutations in *FSHB/Fshb* or in the FSH receptor gene lead to amenorrhea and sterility in females (Kumar *et al.* 1997, Abel *et al.* 2000). Effects in males are species specific, as male *Fshb*-knockout mice are oligozoospermic but still fertile (Kumar *et al.* 1997), whereas men with inactivating mutations in the *FSHB* gene are azoospermic (Layman 2000).

According to current models, *Fshb* expression is stimulated by intra-pituitary activins (Kumar *et al.* 2003, Fortin *et al.* 2015) and hypothalamic gonadotropin-releasing hormone (GnRH) (Dalkin *et al.* 2001, Miller *et al.* 2002). Activins belong to the TGFbeta superfamily and specifically regulate FSH production in gonadotropes, without affecting luteinizing hormone (LH) (Ling *et al.* 1986, Vale *et al.* 1986). The mechanisms through which activins regulate FSH production have been well

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delineated *in vitro* and *in vivo*, at least in the mouse (Dupont *et al*. 2003, Suszko *et al*. 2005, Lamba *et al*. 2010, Fortin *et al*. 2014, 2015, Li *et al*. 2016, 2018). Activins bind to type II activin receptors on the surface of gonadotrope cells, which leads to the recruitment and phosphorylation of type I activin receptors. Once phosphorylated, these receptors phosphorylate intracellular signaling proteins, SMADs 2 and 3, which then associate with SMAD4 in the cytoplasm, accumulate in the nucleus, and bind the *Fshb* promoter (Bernard 2004, Wang *et al*. 2010). SMADs can also form a complex with the transcription factor forkhead box L2 (FOXL2) to promote *Fshb* transcription (Lamba *et al*. 2009, Tran *et al*. 2011, Fortin *et al*. 2014).

GnRH is a decapeptide secreted in pulses from the hypothalamus and regulates both FSH and LH production. How GnRH regulates FSH production is presently unresolved. Several transcription factors, including cAMP response element-binding protein (CREB), CREB-binding protein (CBP), as well as activator protein 1 (AP1) transcription factors have been implicated *in vitro* (Liu *et al*. 2002, Coss *et al*. 2004, 2007, Thompson *et al*. 2013). *In vivo* studies both support (Xie *et al*. 2015, Jonak *et al*. 2017, 2018) and refute (Huang *et al*. 2001, Miller *et al*. 2012) roles for these proteins. One study suggested that histone deacetylases (HDACs) tonically repress the *Fshb* gene and that GnRH relieves this repression by inducing HDAC phosphorylation and nuclear export (Lim *et al*. 2007).

There are four classes of HDACs, though only the first two have been implicated in *Fshb* expression. Class I HDACs are mostly nuclear, while class II HDACs can shuttle between the cytoplasmic and nuclear compartments based on their post-translational modifications (de Ruijter *et al*. 2003). HDACs are generally thought to inhibit gene transcription through at least two mechanisms. First, they can deacetylate histone tails, leading to chromatin compaction (Vidali *et al*. 1978). Second, HDACs can complex with transcriptional repressors that further prevent binding of transcription initiation factors (Glass & Rosenfeld 2000).

In the context of our studies, we attempted to replicate earlier observations that HDACs repress *Fshb* transcription (Lim *et al*. 2007). We were, however, unsuccessful. Rather than stimulating *Fshb* expression, HDAC inhibitors impaired both basal and GnRH induction of the gene in immortalized gonadotrope-like cells. Given that basal *Fshb* is stimulated by endogenous activin-like signaling (Pernasetti *et al*. 2001, Jacobs *et al*. 2003, Fortin *et al*. 2015), we examined the role of HDACs in activin action. Similar to the effects on GnRH induction, HDAC inhibition impaired activin-induced *Fshb* expression in gonadotrope-like cells as well as in primary murine gonadotrope cells. These results challenge a role for HDAC inhibition in activin- or GnRH-stimulated FSH synthesis.

Materials and methods

Reagents

M199 medium with Hank's salt (M7653), collagenase (C0130), pancreatin (P3292), SB431542 (S4317), GnRH (LH releasing hormone, L8008), and trichostatin A (T8552, CAS 5888-19-16) were obtained from Sigma Aldrich. Entinostat (S1053, CAS 209783-80-2), MC1568 (S1484, CAS 852475-26-4), and TMP299 (S7324, CAS 1314890-29-3) were obtained from SelleckChem. EvaGreen (ABMMastermix-S) was from Diamed (Mississauga, ON, Canada). RNasin (0000183771), Moloney murine leukemia virus reverse transcriptase (MMLV RT, 0000172807), DNase (0000156360) and random hexamer primers (0000184865) were from Promega Corporation. TRIzol reagent (15596026), fetal bovine serum (FBS, 10438026) and horse serum (16050122) were obtained from Life Technologies. Deoxynucleotide triphosphates (dNTPs, 800-401-TL), Hank's Balanced Salt Solution media (HBSS, 311-511-CL) and DMEM (319-005-CL) were from Wisent Inc. (St-Bruno, QC, Canada). Recombinant activin A (338-AC-050) was obtained from R&D Systems. Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Total RNA Mini Kit (FA32808-PS) was from Geneaid (New Taipei City, Taiwan). Polyethylenimine (PEI, 23966) was from Polysciences Inc (Warrington, PA, USA).

Cell culture

LβT2 cells (Alarid *et al*. 1996) were provided by Dr Pamela Mellon (University of California, San Diego, CA, USA). Cells were maintained in DMEM (4.5 g/L glucose, with L-glutamine and sodium pyruvate) containing 10% (v/v) FBS. Authenticity of the cells was confirmed by activin treatment, which stimulated *Fshb* mRNA expression (Bernard 2004, Wang *et al*. 2010). LβT2 cells are the only immortalized cell line known to produce *Fshb* basally and in response to activins. Cells were seeded in six-well plates at a density of 2,000,000 cells/well, and in 12-well plates at a density of 1,000,000 cells/well for time-course experiments (between passages 7 and 15). Once they reached 70–80% confluency, cells were serum-starved overnight, and treated in the morning with the indicated

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compounds in serum-free conditions for 6 h: activin A (1 nmol/L), TSA (45 or 331 nmol/L), entinostat (MS-275, 42.5 µmol/L), MC1568 (2.5 µmol/L) and/or TMP269 (3.9 µmol/L) in serum-free medium. The IC_{50} of the different HDAC inhibitors are TSA, ~1.8 nmol/L (Vigushin *et al*. 2001); entinostat, ~1.7 µmol/L (Tatamiya *et al*. 2004); MC1568, ~0.10 µmol/L (Mai *et al*. 2005); and TMP269, ~0.16 µmol/L (Lobera *et al*. 2013). The concentrations of 45 nmol/L, 42.5 µmol/L, 2.5 µmol/L and 3.9 µmol/L for TSA, entinostat, MC1568 and TMP269 are all equal to 25-fold their respective IC_{50}. These concentrations were used to balance between maximal efficacy and off-target effects. The concentration of 331 nmol/L for TSA was sometimes used to allow comparison between our study and previous publications (Lim *et al*. 2007, Oride *et al*. 2014). For time-course experiments, cells were starved overnight and RNA was extracted from cells at 2, 6 and 24 h post treatment (in serum-free conditions). For GnRH induction studies, cells were incubated with GnRH (10 nmol/L) in the presence or absence of TSA (331 nmol/L) for 2 h, followed by a change to medium without GnRH (in the continued presence or absence of TSA) for an additional 2 h.

Pituitaries from 8-week-old C57BL/6 male mice were extracted and dispersed as previously described (Ho *et al*. 2011). Between 250,000 and 400,000 cells/well were seeded in 48-well plates. Cells were cultured for 36 h, after which they were treated with vehicle, TSA (45 or 331 nmol/L), entinostat (42.5 µmol/L) or MC1568 (2.5 µmol/L) in the presence or absence of activin A (1 nmol/L) for 6 h in M199 medium containing 2% (v/v) FBS. Some wells were treated with SB431542 (1 or 10 µmol/L), an activin type I receptor inhibitor (Laping *et al*. 2002). For the entinostat dose–response curve, concentrations of 0, 2, 20, 50, 100 and 200 µmol/L were used (in the presence or absence of activin A). Animal experiments were conducted in accordance with provincial and federal guidelines and were approved by the McGill University and Goodman Cancer Centre Facility Animal Care Committee (Animal Use Protocol #5204).

Protein extraction and western blot

Total protein lysates were extracted as previously described (Turgeon *et al*. 2017). For cytoplasmic and nuclear protein extraction, cells were washed in cold PBS, resuspended in 0.6 mmol/L EDTA in cold PBS using a cell scraper and centrifuged at 4°C. The pellet was incubated in cold buffer A (10 mmol/L Tris, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT) containing protease and phosphatase inhibitors for 15 min, following which 10% (v/v) Nonidet P-40 was added. The pellet was vortexed, left on ice for 1 min, and centrifuged at 12,000***g*** for 5 s, leaving the cytoplasmic proteins in the supernatant. The remaining pellet was washed three times with additional cold buffer A. After the third wash, cold buffer B (20 mmol/L Tris, 400 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT) containing protease and phosphatase inhibitors was added and the sample was left at 4°C for 1 h on a rotator. This was followed by centrifugation at 4°C at 12,000***g*** for 5 min. The supernatant contained the nuclear proteins.

Protein concentration was measured using the Pierce BCA protein assay kit (23227, ThermoFisher Scientific), following the manufacturer's instructions. Between 10 and 20 µg of each protein lysate was denatured at 95°C for 10 min prior to being resolved by SDS-PAGE. Proteins were transferred to Protran nitrocellulose membranes (NBA083C001EA; Perkin Elmer), blocked in 5% BSA (w/v) in Tris-buffered saline (TBS; 150 mmol/L NaCl, 10 mmol/L Tris (pH 8.0)) containing 0.05% (v/v) Tween 20 (TBST) and incubated in the indicated primary Ab in 5% (w/v) milk in TBST overnight at 4°C with agitation. The rabbit anti-calnexin (1:1000, sc-11397) and mouse anti-acetylated alpha-tubulin (1:5000, sc-23950) were purchased from Santa Cruz BioTechnology Inc. The mouse anti-nucleoporin (1:3000, 610498) was purchased from BD Transduction Laboratories. The polyclonal rabbit anti-phospho-SMAD2 (1:2000, 3101) was purchased from Cell Signaling. The rabbit polyclonal anti-SMAD2/3 (1:2000, 07-408) and the anti-acetyl-Histone H4 Lys 12 (H4K12; 1:2000, 04-119) were purchased from Millipore. The mouse anti-alpha-tubulin (1:10000, Ab7291) was purchased from Abcam. The next day, the membranes were washed in TBST and subsequently incubated in horseradish peroxidase–conjugated rabbit or mouse secondary Ab (1:5000, goat anti-mouse 170-6516, goat anti-rabbit 170-6515; Bio-Rad Laboratories) in 5% (w/v) milk in TBST for 1 h at room temperature. Membranes were washed in TBST, enhanced chemiluminescence substrate (ECL; NEL105001 PerkinElmer) was applied, and membranes were digitally visualized with an Amersham Imager 600 (G&E Healthcare). Membranes were stripped in 0.2 M sodium hydroxide solution, washed in TBST and new primary antibody was applied.

For protein quantification, the Bio-Rad QuantityOne software was used, and phospho-SMAD2 levels were normalized to total SMAD2 levels.

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Validation of HDAC inhibitors

LβT2 cells were plated in six-well plates at a density of 2,000,000 cells/well. The next day, cells were serum-starved overnight, and treated with vehicle, TSA (45 nmol/L), entinostat (42.5 µmol/L) or TMP269 (3.9 µmol/L). Proteins were extracted as described earlier.

C2C12 cells were provided by Dr Simon Rousseau (McGill University, Montreal, Canada). Cells were maintained in growth medium (GM; DMEM supplemented with 10% FBS (v/v)). Cells were plated in 24-well plates at a density of 75,000 cells/well. On the next day, cells were treated in GM with vehicle, MC1568 (2.5 µmol/L) or TSA (45 nmol/L) for 24 h. Then, medium was switched to differentiation medium (DM; DMEM supplemented with 2% horse serum (v/v)) containing vehicle, MC1568, or TSA using the same concentrations. An additional control was maintained on GM. After another 24 h of treatment, RNA was harvested, and gene expression studies were performed as described below.

Reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from cell lines with TRIzol following the manufacturer's guidelines. RNA from primary cells was extracted using the Geneaid Total RNA Mini kit. Between 500 ng (12-well plates) and 1 µg (6 well plates) of RNA were reverse-transcribed for LβT2 cells, 500 ng for C2C12 cells and 100 ng for primary cells. RNA concentrations were determined by NanoDrop. Reverse transcription was performed as previously described (Bernard 2004), using MMLV reverse transcriptase and random hexamer primers. The obtained cDNA was then used for qPCR analysis on a Corbett Rotorgene 600 instrument (Corbett Life Science) using EvaGreen and primers listed in Table 1. mRNA levels of target genes were determined using the $2^{-\Delta\Delta CT}$ method. Ribosomal protein L19 (*Rpl19*) was used for normalization. All primers were validated for efficiency and specificity.

Promoter-reporter assay

For promoter-reporter assays, LβT2 cells were seeded in 48-well plates at a density of 150,000 cells/well. Approximately 24 h after seeding, the cells were transfected with 225 ng/well of CAGA-luciferase reporter plasmid (Dennler *et al.* 1998) using Lipofectamine 2000 (11668019, ThermoFisher Scientific) overnight. The next day, cells were starved in serum-free medium, following which they were treated with vehicle (DMSO), TSA (45 nmol/L), entinostat (42.5 µmol/L), MC1568 (2.5 µmol/L) or TMP269 (3.9 µmol/L), in the presence or absence of activin A (1 nmol/L) for 2, 6 and 24 h.

HEK293 cells were provided by Dr Terry Hébert (McGill University, Montreal, Canada). Cells were maintained in DMEM supplemented with 5% FBS (v/v), and seeded in 48-well plates at a density of 50,000 cells/well. Approximately 24 h after seeding, cells were transfected with 225 ng/well of −326/+1 porcine *Fshb*-promoter-luciferase reporter plasmid (Lamba *et al.* 2009) in the presence or absence of 4.15 ng/well of a FOXL2 expression construct (Lamba *et al.* 2009), using polyethylenimine (PEI) for 2 h in serum-free medium. After this incubation, medium was replaced with fresh culture medium (5% FBS (v/v)). The next day, cells were starved in serum-free medium, following which they were treated with vehicle (DMSO) or TSA (45 nmol/L) in the presence or absence of activin A (1 nmol/L) for 6 h.

For all luciferase assays, lysates were prepared and collected as in the study by Wang *et al.* (2010). Luciferase assays were performed on an Orion II microplate luminometer (Berthold Detection Systems, Oak Ridge, TN, USA). All conditions were performed in duplicate wells in four independent experiments.

Table 1 qPCR primers.

Gene	Primer sequence	
	Forward	Reverse
Rpl19	CGGGAATCCAAGAAGATTGA	TTCAGCTTGTGGATGTGCTC
Fshb	GTGCGGGCTACTGCTACACT	CAGGCAATCTTACGGTCTCG
Lhb	AGCAGCCGGCAGTACTCGGA	ACTGTGCCGGCCTGTCAACG
Cga	TCCCTCAAAAAGTCCAQGAGC	GAAGAGAATGAAGAATATGCAG
Hsd17b1	GTTATGAGCAAGCCCTGAGC	AAGCGGTTCGTGGAGAAGTA
Myog	GCACTGGAGTTCGGTCCCA	GATGGACGTAAGGGAGTGCAGA
Fos	GGAGCTGACAGATACACTCCAA	GAGGCCACAGACATCTCCTC
Egr1	GAGCGAACAACCCTATGAGC	GAGTCGTTTGGCTGGGATAA

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Statistical analysis

Data were analyzed by one-way or two-way ANOVA, followed by *post hoc* Holm–Sidak multiple comparison test. Statistical analyses were performed using GraphPad Prism 7. Results were considered statistically significant when $P<0.05$.

Results

HDAC inhibition impairs *Fshb* expression in LβT2 cells

According to previous reports (Lim *et al*. 2007, Oride *et al*. 2014, Mijiddorj *et al*. 2017), *Fshb* mRNA expression was significantly increased in LβT2 and αT3-1 gonadotrope-like cell lines treated with 20–200 ng/mL (66.1–661 nmol/L) TSA. This was a remarkable result, particularly for αT3-1 cells, which were never before shown to express *Fshb*. However, our efforts to replicate these results using the published assay conditions were unsuccessful, as TSA failed to stimulate *Fshb* mRNA levels in either cell line in our hands (Fig. 1 and data not shown). In contrast, GnRH-stimulated *Fshb* expression in LβT2 cells was robustly inhibited by TSA (Fig. 1A). GnRH induction of other known targets, *Fos* and *Egr1*, were not impaired (Fig. 1B and C); TSA actually potentiated GnRH-stimulated *Egr1* expression. TSA also suppressed basal *Fshb* mRNA levels (although it was not statistically significant) in these cells (Fig. 1A), which are dependent on the actions of an endogenous activin-like ligand (Pernasetti *et al*. 2001, Jacobs *et al*. 2003, Fortin *et al*. 2015). TSA also significantly decreased *Fshb* mRNA levels following exogenous activin A stimulation (Fig. 1D). TSA-mediated inhibition of basal and activin A-stimulated *Fshb* mRNA expression was observed whether cells were treated for 2, 6 or 24 h (Fig. 1E). The effects were specific, as basal and activin A-stimulated *Lhb* expressions were not significantly affected by TSA at 2 or 6 h (Fig. 1F). An impairment was observed in the activin-treated condition at 24 h, which may be a side effect of prolonged exposure to TSA, which can have cytotoxic effects (Wharton *et al*. 2000).

A class I, but not class II, HDAC inhibitor recapitulates the effects of TSA

TSA targets a wide range of HDACs. We therefore treated LβT2 cells with class-specific inhibitors to better refine the relevant class(es) of HDACs in gonadotrope-like cells. Entinostat (MS-275), a class I HDAC inhibitor

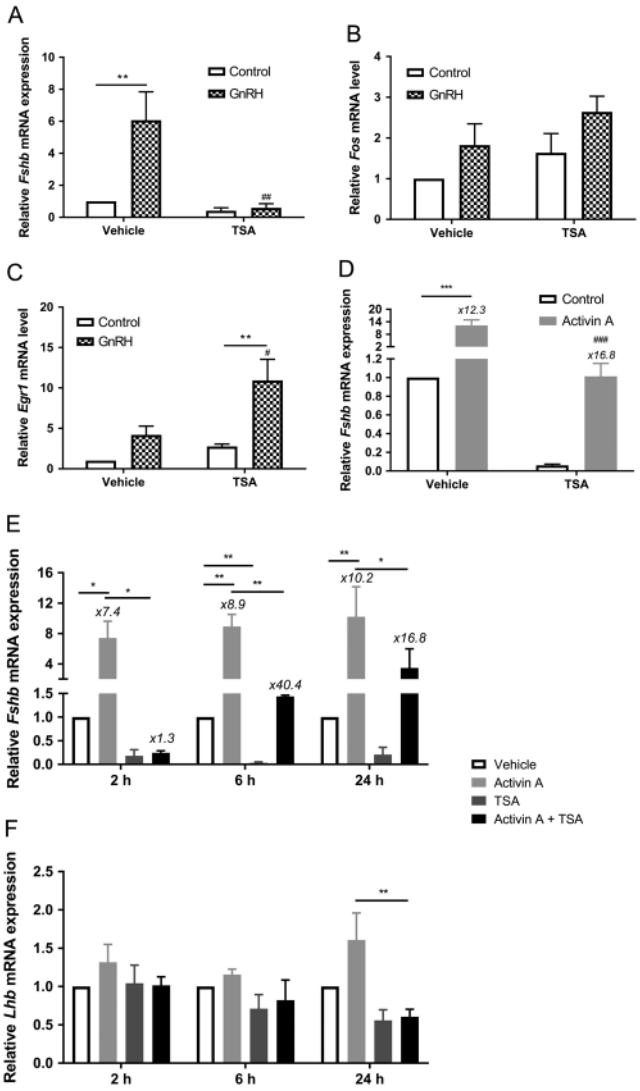

Figure 1
HDAC inhibition impairs basal, GnRH-, and activin A-induced *Fshb* expression. (A, B and C) LβT2 cells were treated for 2 h with GnRH (10 nmol/L) in the presence or absence of TSA (331 nmol/L), followed by incubation in GnRH-free medium for an additional 2 h, in the presence or absence of TSA. $n = 3$ independent experiments. (A) *Fshb*, (B) *Fos*, and (C) *Egr1* mRNA levels were measured by RT-qPCR and normalized to the housekeeping gene *Rpl19*. (D) LβT2 cells were treated for 6 h with activin A (1 nmol/L) in the presence or absence of TSA (331 nmol/L). $n = 5$ independent experiments. *Fshb* mRNA levels were measured by RT-qPCR and normalized to the housekeeping gene *Rpl19*. (E) and (F) Cells were treated as in panel D and RNA was collected after 2, 6 or 24 h of treatment. $n = 3$ independent experiments. *Fshb* and *Lhb* mRNA levels were measured by RT-qPCR and normalized to the housekeeping gene *Rpl19*. For each time point, data were normalized to the control condition. In all panels, bars represent mean values (± S.E.M.). Activin A's fold induction is indicated above the appropriate bars. Data were analyzed by two-way ANOVAs followed by a *post-hoc* Holm–Sidak multiple comparison test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ when comparing activin A vs control; $^{\#\#\#}P < 0.001$ when comparing TSA vs corresponding vehicle condition.

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(Khan *et al.* 2008), significantly impaired activin A-induced, but not basal, *Fshb* expression (Fig. 2A). On the other hand, neither MC1568 (Fig. 2A) nor TMP269 (Fig. 2B), two class II HDAC inhibitors (Mai *et al.* 2005, Lobera *et al.* 2013), impacted *Fshb* expression.

To confirm the activity of all of the HDAC inhibitors, we examined known markers and targets: acetylated histone 4 lysine 12 (H4K12) for TSA and entinostat (Paradis & Hales 2015) and myogenin for MC1568 (Nebbioso *et al.* 2009). There are no well-described targets for TMP269, so we assessed its effects on α-tubulin acetylation. Both TSA and entinostat induced acetylation of H4K12 in LβT2 cells (Fig. 2C), while TMP269 increased levels of acetylated α-tubulin (Fig. 2D). As previously reported, MC1568 (and TSA) blunted the upregulation of myogenin expression (encoded by *Myog*) following differentiation of C2C12 myoblasts (Fig. 2E).

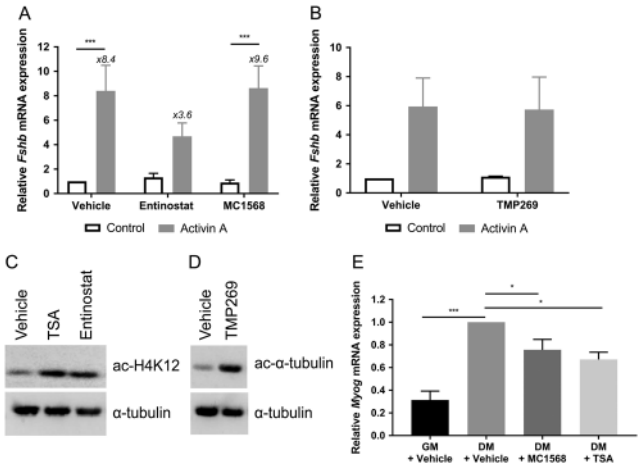

Figure 2
Class I, but not class II, HDAC inhibition inhibits activin A-induced *Fshb* mRNA levels. (A) LβT2 cells were treated for 6 h with a class I (entinostat) or a class II HDAC inhibitor (MC1568), at a concentration of 42.5 µmol/L and 2.5 µmol/L, respectively ($n = 4$). (B) LβT2 cells were treated in separate experiments for 6 h with TMP269, a second class II HDAC inhibitor, at a concentration of 3.9 µmol/L ($N = 2$). In (A) and (B), *Fshb* mRNA levels were measured by RT-qPCR and normalized to the housekeeping gene *Rpl19*. Activin A's fold induction is indicated above the appropriate bars in panel A. (C and D) LβT2 cells were treated for 6 h with vehicle (DMSO), TSA (45 nmol/L), entinostat (42.5 µmol/L), or TMP269 (3.9 µmol/L). Total protein lysates were extracted and levels of acetylated H4K12 (panel C), acetylated α-tubulin (panel D), and α-tubulin (panels C and D) were analyzed by western blot. (E) C2C12 cells were treated for 24 h with vehicle (DMSO), MC1568 (2.5 µmol/L), or TSA (45 nmol/L) in GM. After incubation, cells were grown for another 24 h in GM or differentiation medium (DM) in the presence of vehicle (DMSO), MC1568 (2.5 µmol/L), or TSA (45 nmol/L). *Myog* mRNA levels were measured by RT-qPCR and normalized to the housekeeping gene *Rpl19*; data were normalized to the vehicle-treated DM condition ($n = 3$). Bars represent mean values (± S.E.M.). Data were analyzed by two-way ANOVA (panels A and B) or one-way ANOVA (panel E) followed by *post-hoc* Holm–Sidak multiple comparison test; $^{*}P < 0.05$, $^{***}P < 0.001$.

HDAC inhibitors impair *Fshb* expression in primary pituitary cultures

The discrepancy between our results and those of earlier reports (Lim *et al.* 2007, Oride *et al.* 2014) could have derived from batch to batch differences in LβT2 cells. To address this possibility, we investigated the effects of HDAC inhibitors on *Fshb* expression in murine primary pituitary cell cultures. As we observed in LβT2 cells, basal and activin A-induced *Fshb* mRNA levels were reduced by 95 and 70%, respectively, in the presence of 331 nmol/L TSA compared to control (Fig. 3A). In contrast, neither *Lhb* nor *Cga* mRNA levels were affected by TSA (Fig. 3B and C). To better compare TSA with the class-specific inhibitors, we also treated primary pituitary cells with 45 nmol/L TSA, instead of 331 nmol/L (see Methods; all inhibitors were used at a concentration equivalent to 25-fold their IC_{50}). The lower concentration of TSA affected the amplitude, but not the directionality nor the significance of its inhibitory effects on *Fshb* (Figs 3A vs 4A). The class I (entinostat), but not class II HDAC inhibitor (MC1568), suppressed basal and activin A-induced *Fshb* expression in primary cells (Fig. 4A). Next, we treated primary cells with 0, 2, 20, 50, 100 and 200 µmol/L of entinostat. Entinostat affected activin A-induced *Fshb* expression

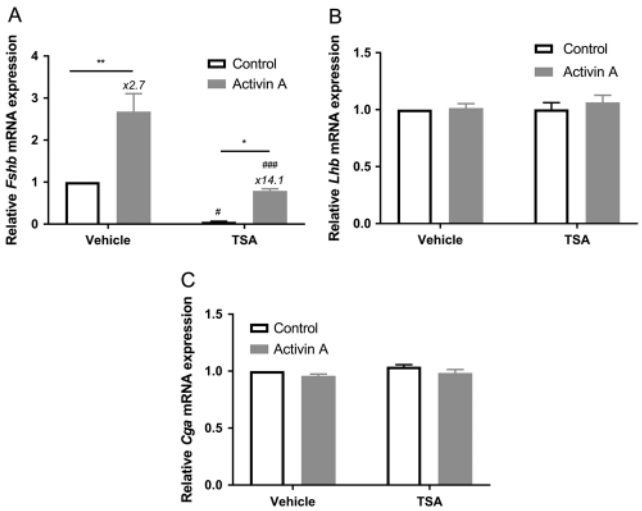

Figure 3
Fshb expression in primary pituitary cells is reduced by HDAC inhibition. Pituitaries from 8-week-old male mice were dispersed and cultured. After 2 days in culture, cells were treated with activin A (1 nmol/L), TSA (331 nmol/L), or both, for 6 h. mRNA levels of gonadotropin subunits (*Fshb* (A), *Lhb* (B), and *Cga* (C)) were measured by RT-qPCR and normalized to the housekeeping gene *Rpl19*. Bars represent mean values (± S.E.M.) of $n = 3$ independent experiments. Activin A's fold induction is indicated above the appropriate bars. Two-way ANOVA followed by a *post-hoc* Holm–Sidak multiple comparison test was used. $^{*}P < 0.05$; $^{**}P < 0.01$ when comparing activin A vs control; $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$ when comparing TSA vs corresponding vehicle condition.

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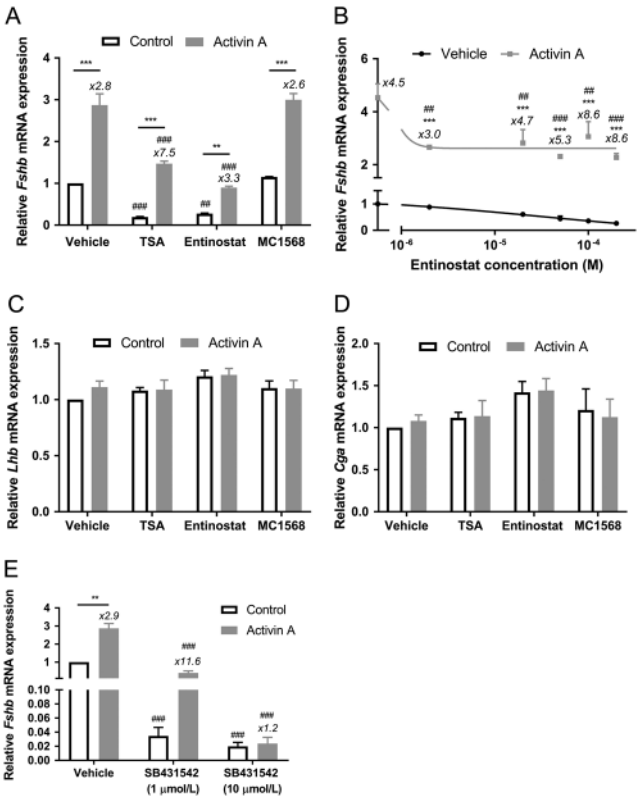

Figure 4
Class I, but not class II, HDAC inhibition suppresses *Fshb* expression in primary pituitary cultures. Pituitaries from 8-week-old male mice were dispersed and cultured. After 2 days in culture, cells were treated with TSA (45 nmol/L in (A, C and D)), entinostat (42.5 µmol/L in (A, C and D) or a range of concentrations in (B)), MC1568 (2.5 µmol/L in (A, C and D)), or SB431542 (1 or 10 µmol/L in (E)), in the presence or absence of activin A (1 nmol/L), for 6 h. mRNA levels of gonadotropin subunits (*Fshb* (A, B and E), *Lhb* (C), and *Cga* (D)) were measured by RT-qPCR and normalized to the housekeeping gene *Rpl19*. Bars represent mean values (± S.E.M.) of $n = 3$ independent experiments, or $n = 2$ independent experiments for the dose-response curve in panel B. Activin A's fold induction is indicated above the appropriate bars or data points. Data were analyzed by two-way ANOVA followed by a *post-hoc* Holm–Sidak multiple comparison test. $^{***}P < 0.001$ when comparing activin A vs control; $^{\#\#\#}P < 0.001$ when comparing the inhibitor vs the corresponding vehicle condition.

at a concentration as low as 2 µmol/L (equivalent to its IC_{50}; Fig. 4B). Higher concentrations did not affect the amplitude of this effect. Basal *Fshb* was reduced in a more dose-dependent manner, although the differences were not statistically significant. *Lhb* and *Cga* mRNA levels were not inhibited by TSA, entinostat, or MC1568 (Fig. 4C and D). The apparent induction of *Cga* expression by entinostat was not statistically significant ($P = 0.067$).

A consistent effect of TSA and entinostat was to increase the apparent potency of exogenous activin A in both LβT2 and primary cells. That is, the fold induction by activin A was often increased in the presence of HDAC inhibitors. This effect was principally driven by

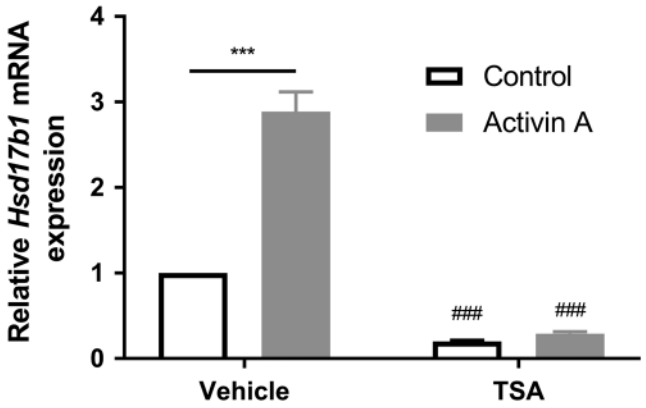

Figure 5
HDAC inhibition impairs *Hsd17b1* mRNA expression. LβT2 cells were treated for 6 h with activin A (1 nmol/L) in the presence or absence of TSA (331 nmol/L). *Hsd17b1* mRNA levels were measured by RT-qPCR and normalized to the housekeeping gene *Rpl19*. Bars represent mean values (± S.E.M.) of $n = 3$ independent experiments. Data were analyzed by two-way ANOVA followed by *post-hoc* Holm–Sidak multiple comparison test. $^{**}P < 0.01$ when comparing activin A vs control; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ when comparing TSA vs corresponding vehicle condition.

the decreases in basal *Fshb* expression. It is not clear how to interpret these results, as we have seen similar results with direct antagonists of activin signaling. For example, we treated primary pituitary cells with 1 or 10 µmol/L of SB431542, an activin type I receptor inhibitor (Fig. 4E). At 10 µmol/L, both basal and activin A-induced *Fshb* mRNA expression levels were severely impaired. However, at a sub-optimal concentration (1 µmol/L), basal *Fshb* was impaired, while the fold (but not absolute) activin A response was increased from 2.9 to 11.6. At a minimum, these data suggest that the amount of inhibitor required to antagonize endogenous activin-like signaling is less than that needed to inhibit exogenous activin A.

TSA impairs expression of another activin target, *Hsd17b1*

Results from both immortalized and primary gonadotrope cells indicated that inhibition of class I HDACs impaired activin A-stimulated *Fshb* expression. The results did not indicate, however, whether these effects were gene specific or reflected a generalized antagonism of activin-like signaling. We therefore examined the effects of TSA on a second activin A-responsive gene in LβT2 cells, 17β-hydroxysteroid dehydrogenase type I (*Hsd17b1*) (Bak *et al.* 2009). Similar to the case with *Fshb*, TSA greatly attenuated basal and activin A-induced *Hsd17b1* expression (Fig. 5).

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TSA does not impair activin A-induced SMAD signaling

The above results suggested that TSA (and entinostat) somehow antagonized activin action but did not indicate how. A key step in activin signaling is phosphorylation of receptor-regulated SMAD proteins (SMAD2 and SMAD3) by activin type I receptors. Phosphorylated SMADs then partner with SMAD4 and accumulate in the nucleus. To investigate whether or not TSA impaired this part of the pathway, we first measured levels of phosphorylated SMAD2. However, TSA appeared to enhance, rather than inhibit, activin A induction of SMAD2 phosphorylation in LβT2 cells (Fig. 6A). Activin A-stimulated nuclear accumulation of pSMAD2 was also unimpaired by TSA (Fig. 6B). These data suggested that TSA did not affect the ability of activin receptors to bind ligand or to activate intracellular signaling.

Activin A induction of *Fshb* mRNA expression depends on SMAD3 and SMAD4. To determine whether TSA or other HDAC inhibitors impaired SMAD3/4 signaling, we examined their effects on activin A induction of the SMAD3/4-dependent promoter-reporter CAGA-luc (Dennler *et al.* 1998) in LβT2 cells. In vehicle-treated conditions (DMSO alone), activin A robustly induced luciferase activity after 2, 6 and 24 h, though statistical significance was only reached at the latter time points (Fig. 7). TSA, at 45 nmol/L, did not suppress basal reporter activity, but impaired the activin A response by ~30–50% at 2 h, though not statistically significantly (Fig. 7A). This apparent reduction was reversed by 6 h. At 24 h, activin A-stimulated reporter activity was enhanced by TSA by about two-fold. We obtained comparable data when using 331 nmol/L TSA (data not shown). Entinostat showed similar effects to those of TSA: impaired activin stimulation at 2 h, no effect at 6 h and a robust rebound effect at 24 h, with a ~12-fold increase in activin responsiveness compared to DMSO-treated cells (Fig. 7B). The class II-specific inhibitors had no effects at any of the time points investigated (Fig. 7C and D). Collectively, these data suggest that any inhibitory effects of HDAC inhibitors on SMAD3/4-signaling are short-lived. If anything, these inhibitors appeared to enhance SMAD3/4 signaling.

TSA does not impair FOXL2-mediated *Fshb* transcription

Activin induction of *Fshb* is FOXL2 dependent (Lamba *et al.* 2009). We therefore examined whether HDAC inhibition affected FOXL2 activity. We previously reported that a porcine *Fshb*-luciferase reporter was unresponsive to activin A in heterologous cells (Lamba *et al.* 2009). However, co-transfection of a FOXL2 expression vector is sufficient to confer activin sensitivity. We repeated those results here in HEK293 cells (Fig. 8). TSA potentiated the effects of FOXL2 in the presence and absence of activin A, suggesting that HDAC inhibition does not impair FOXL2 function.

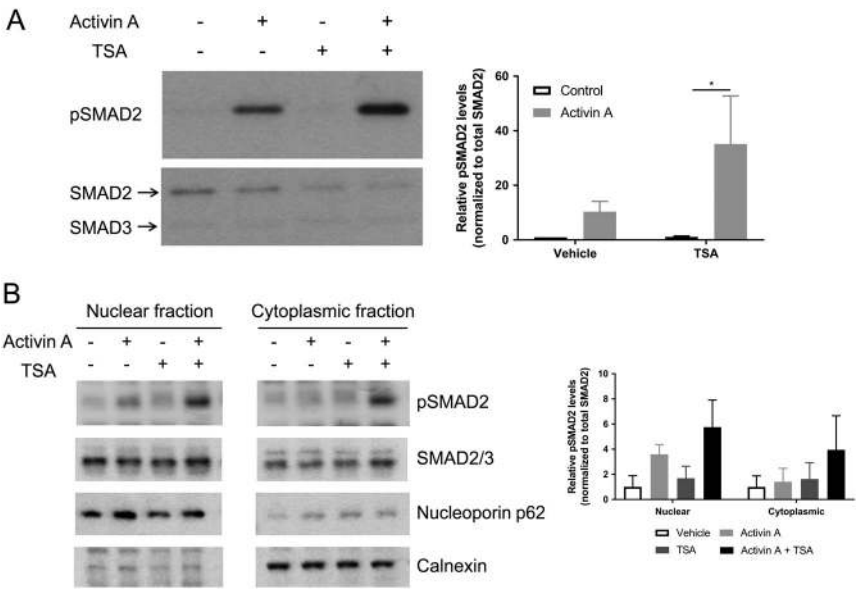

Figure 6
HDAC inhibition does not block activin A-induced SMAD2 phosphorylation or nuclear import. (A) LβT2 cells were treated for 6 h with activin A (1 nmol/L) in the presence or absence of TSA (331 nmol/L). Total lysates were extracted and levels of phospho-SMAD2 and total SMAD2 were analyzed by western blot. A representative blot is shown. The data at the right represent the mean (± S.E.M.) of $n = 4$ independent experiments, and were analyzed by two-way ANOVA, followed by *post-hoc* Holm–Sidak multiple comparison test. *$P < 0.05$ when comparing activin A vs control. (B) A similar experiment was conducted, but nuclear and cytoplasmic protein fractions were prepared. Levels of pSMAD2, nucleoporin p62 (nuclear marker), and calnexin (cytoplasmic marker) were assessed by western blot. Both cytoplasmic and nuclear fractions were run on the same gel, and exposure times were the same between both compartments (intervening lanes were cropped for purposes of figure preparation). For protein quantification, phospho-SMAD2 levels were normalized to total SMAD2 levels, using the Bio-Rad QuantityOne software.

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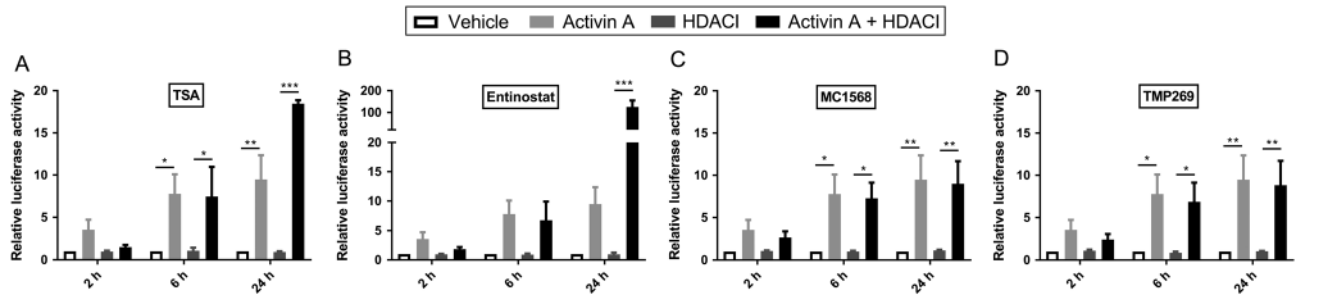

Figure 7
HDAC inhibition does not significantly affect SMAD3/4 signaling over short periods of time, and promotes it following longer incubation times. LβT2 cells were transfected with 225 ng of the CAGA-luc reporter plasmid, followed by treatment for 2, 6, or 24 h with (A) TSA (45 nmol/L), (B) MS-275 (42.5 μmol/L), (C) MC1568 (2.5 μmol/L), or (D) TMP269 (3.9 μmol/L) in the presence or absence of activin A (1 nmol/L). For each time point, values were normalized to the vehicle, control-treated condition. Bars represent mean values (± S.E.M.) of $n = 4$ independent experiments. Data were analyzed by two-way ANOVA followed by *post-hoc* Holm–Sidak multiple comparison test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Discussion

We observed that TSA robustly inhibited basal as well as GnRH- and activin A-stimulated *Fshb* expression. Our results were surprising on at least two counts. First, they contradict those of earlier studies. Second, histone acetylation is usually associated with chromatin de-compaction and promotion of transcription. However, it is now clear that histone deacetylases mediate other functions in addition to histone deacetylation. We explored some of these putative functions in an effort to provide a mechanistic explanation for our results.

We investigated the potential role for non-histone substrate acetylation (Juan *et al.* 2000, Zhang *et al.* 2003). FOXL2, an essential transcription factor for *Fshb* expression (Tran *et al.* 2011, Tran *et al.* 2013, Fortin *et al.* 2014, Li *et al.* 2018), undergoes several post-translational modifications, including acetylation (Georges *et al.* 2011). To our knowledge, the role of FOXL2 acetylation in its transactivation function has not been investigated. However, acetylation of other forkhead proteins can impair their binding to DNA (Daitoku *et al.* 2011). We therefore asked whether HDAC inhibitors might enhance FOXL2 acetylation and block the protein's binding to the *Fshb* promoter. TSA induced FOXL2 acetylation (data not shown), but only at concentrations that exceeded those needed to inhibit *Fshb* expression. Entinostat did not alter FOXL2 acetylation (data not shown). Finally, TSA did not impair FOXL2-dependent induction of a porcine *Fshb*-luciferase reporter in heterologous cells (Fig. 8). We did not investigate FOXL2 recruitment to the *Fshb* promoter in TSA- or entinostat-treated LβT2 cells, as we lack a ChIP-grade antibody for FOXL2. Nonetheless, the available data suggest that the effects of TSA and entinostat are likely to be FOXL2 independent.

A second mechanism by which HDACs are thought to promote transcription is through the control of elongation of paused genes. On the promoters of such genes, negative elongation factor (NELF) interacts with RNA Poll II to prevent the elongation phase from taking place (Greer *et al.* 2015). HDACs inhibit this interaction, releasing the paused state and promoting elongation. As a consequence, HDAC inhibitors can be used to maintain genes in a paused state (Greer *et al.* 2015). It is not yet known whether *Fshb* is a paused gene. To begin to address this question, we inhibited HSP90, a chaperone protein required for the Pol II and NELF interaction

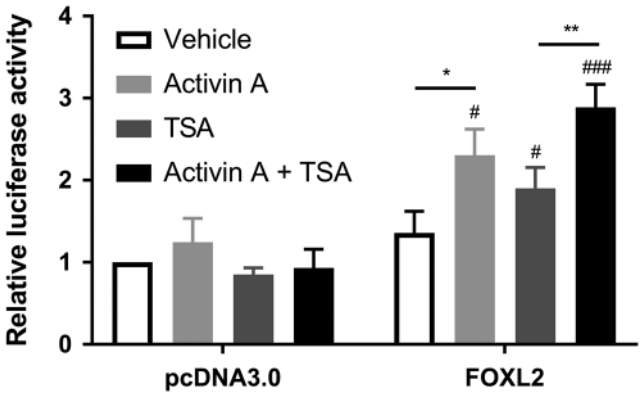

Figure 8
HDAC inhibition does not impair FOXL2 regulation of the porcine *Fshb* promoter. HEK293 cells were transfected with 225 ng of a porcine *Fshb*-promoter–luciferase reporter plasmid, along with 4.15 ng of an empty vector (pcDNA3.0) or a FOXL2 expression vector. Cells were treated for 6 h with TSA (45 nmol/L) in the presence or absence of activin A (1 nmol/L). Values were normalized to the vehicle, control-treated condition (empty vector). Bars represent mean values (± S.E.M.) of $n = 3$ independent experiments. Data were analyzed by two-way ANOVA followed by *post-hoc* Holm–Sidak multiple comparison test. $^{*}P < 0.05$, $^{**}P < 0.01$ when comparing treatments to the vehicle condition; $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ when comparing pcDNA3.0 and FOXL2 for a given treatment condition.

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(Sawarkar *et al*. 2012), using geldanamycin. Assuming that *Fshb* is in a paused state, preventing this interaction should promote elongation and increase mRNA levels. However, we observed a robust inhibition of *Fshb* expression (data not shown), which, at first blush, might suggest that *Fshb* is not paused. However, SMAD signaling is also dependent on HSP90 (Wrighton *et al*. 2008), which confounds a clear interpretation of these data. Thus, whether or not HDAC inhibitors affect *Fshb* expression by impairing transcriptional elongation is unresolved. What is clear, however, is that the HDAC inhibitors did not block SMAD2 phosphorylation (Fig. 6) or SMAD3/4-dependent signaling (Fig. 7). Therefore, impaired *Fshb* (and *Hsd17b1*) expression appears to be SMAD independent.

As our data contrast with those of previously published work, we employed several approaches to uncover the potential sources of variation. First, we used different TSA concentrations. However, *Fshb* was inhibited at both 45 and 331 nmol/L, indicating that the directionality of the effect was not concentration dependent. This is an important consideration as previous studies used between 331 nmol/L and 5 µmol/L (Lim *et al*. 2007, Oride *et al*. 2014, Mijiddorj *et al*. 2017), even though TSA's IC_{50} is in the low nanomolar range (~1.8 nmol/L) (Vigushin *et al*. 2001). Given the high concentrations that we and others used, it is possible that the effects observed stemmed from off-target effects of the drug. Second, we compared different durations of TSA treatment. TSA is a known cell cycle inhibitor (Wharton *et al*. 2000) and previous studies mostly used 24-h treatments (Lim *et al*. 2007, Oride *et al*. 2014, Mijiddorj *et al*. 2017). We treated cells for 2, 6 or 24 h, and *Fshb* expression was inhibited at all time points. Third, we compared TSA from two different vendors. Again, both inhibited *Fshb* expression (data not shown). Fourth, we used four different *Fshb* qPCR primer sets, including those used by the groups mentioned above. All revealed a decrease in *Fshb* mRNA levels following TSA treatment (data not shown). Fifth, we compared different HDAC inhibitors, a hydroxamic acid (TSA) and a benzamide (entinostat), and both impaired *Fshb* expression. Moreover, two different class II inhibitors, MC1568 and TMP269 (the activities of which were confirmed), failed to affect *Fshb* expression. This indicates that the inhibitory effects on *Fshb* expression observed in this study are robust and specifically mediated by class I HDACs.

Collectively, our multi-pronged approach indicates that HDACs play a permissive, rather than inhibitory role in *Fshb* expression *in vitro*. Importantly, the results in immortalized cells were corroborated in murine primary pituitary cultures. Thus, these effects were likely not due to batch-to-batch differences in the cell line and were not specific to immortalized cells. Therefore, our data fail to support a role for HDAC inhibition in GnRH or activin regulation of *Fshb* expression. In contrast, HDAC inhibitors appear to impair activin stimulation of *Fshb* through a SMAD- and FOXL2-independent, but still uncharacterized mechanism.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

G S and D J B were responsible for the experimental design, data analyses, and manuscript preparation. G S conducted most of the experiments. C T conducted the experiments involving GnRH stimulation. All authors approved the final version of the manuscript.

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