International Journal of Biochemistry and Cell Biology 113 (2019) 8–16

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

# Histone deacetylase inhibitors promote *ATP2A3* gene expression in hepatocellular carcinoma cells: p300 as a transcriptional regulator

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ARTICLE INFO

*Keywords:*
SERCA3
Chromatin remodeling
Liver cancer
Sodium butyrate
Trichostatin A

ABSTRACT

Sarco(endo)plasmic reticulum $Ca^{2+}$-ATPases (SERCA) expression is reduced or absent in several types of cancer and cancer cell lines; however, their expression and regulation in hepatocellular carcinoma (HCC) are unknown. Histone deacetylase inhibitors (HDACi) increase SERCA3 mRNA expression in gastric and breast cancer cell lines by increasing H3K9ac and binding of Sp1 and Sp3 transcription factors to the promoter; however, the molecular mechanism is not fully understood. Our results show that *ATP2A3* (SERCA3) gene expression is decreased in human HCC samples and rat HCC AS-30D cells compared to normal liver, and HCC patients with high expression of *ATP2A3* had longer overall survival than those with low expression. Sodium butyrate (NaB) and trichostatin A (TSA) increase SERCA3 mRNA expression in AS-30D cells, whereas SERCA2b mRNA expression did not change. NaB and TSA increase H3K9ac and H3K27ac in two *ATP2A3* promoter regions. Besides, NaB treated cells increased Sp1 and Sp3 occupancy at *ATP2A3* promoter; whereas TSA treated cells showed increased p300 levels at *ATP2A3* promoter. Inhibition of p300 by C646, a specific inhibitor, mitigates SERCA3 mRNA induction by TSA, and reduces more than 70% of basal SERCA3 mRNA expression, suggesting that p300 is important for *ATP2A3* gene transcription in AS-30D cells. Moreover, inhibition of p300 decreases H3K9ac in TSA treated cells. Our results provide evidence of decreased SERCA3 expression in human HCC samples and rat AS-30D cells and a correlation of SERCA3 expression with overall survival in HCC patients. Also, reveal new insights in SERCA3 transcriptional regulation mediated by HDACi.

## 1. Introduction

Calcium ion ($Ca^{2+}$) regulates a broad range of cellular functions like gene expression, cell cycle, muscle contraction, cell growth and differentiation, angiogenesis, and cell death (Roderick and Cook, 2008; Monteith et al., 2007). Several proteins, including $Ca^{2+}$ pumps, exchangers, effectors, channels, among others, modulate $Ca^{2+}$ signaling by increasing and decreasing intracellular $Ca^{2+}$ concentrations in time and cell-specific manner (Berridge et al., 2003). In many diseases like cancer, cells remodel the expression and activity of the $Ca^{2+}$ signaling protein machinery (Roderick and Cook, 2008; Berridge et al., 2003).

Sarco(endo)plasmic reticulum $Ca^{2+}$-ATPases (SERCA) transport $Ca^{2+}$ from the cytosol into the sarco(endo)plasmic reticulum, and have a major role in $Ca^{2+}$ homeostasis (Brini and Carafoli, 2009). There are three SERCA enzymes (SERCA1, 2, and 3) encoded by three different genes: *ATP2A1*, *ATP2A2*, and *ATP2A3* (Brini and Carafoli, 2009; Wuytack et al., 2002). The mRNA from the SERCA genes undergo splicing, generating several isoforms. *ATP2A1* encodes for SERCA1a and SERCA1b, *ATP2A2* encodes SERCA2a, SERCA2b, and SERCA2c, whereas *ATP2A3* encodes six SERCA3 isoforms (a–f) in humans and two isoforms in rats (Brini and Carafoli, 2009; Wuytack et al., 2002).

The evidence suggests that *ATP2A3* gene expression could have a major role in several types of cancer. SERCA3 expression is downregulated in several types of cancer and cancer cell lines such as breast, oral, lung, colon, gastric, and choroid plexus (Endo et al., 2004; Brouland et al., 2005; Gélébart et al., 2002; Papp and Brouland, 2011;

*Abbreviations:* SERCA, sarco(endo)plasmic reticulum $Ca^{2+}$ ATPase; NaB, sodium butyrate; TSA, trichostatin A; HDAC, histone deacetylase; HAT, histone acetyltransferase; H3K9ac, acetylated lysine 9 of histone 3; H3K27ac, acetylated lysine 27 of histone 3; ER, endoplasmic reticulum

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https://doi.org/10.1016/j.biocel.2019.05.014
Received 23 February 2019; Received in revised form 22 May 2019; Accepted 25 May 2019
Available online 27 May 2019

Arbabian et al., 2013; Ait-Ghezali et al., 2014; Contreras-Leal et al., 2016; Flores-Peredo et al., 2016). Recently, we and others have demonstrated that treatment of cancer cell lines with histone deacetylase inhibitors (HDACi), like sodium butyrate (NaB) or trichostatin A (TSA) increases SERCA3 mRNA expression by increasing histone H3 acetylation at the proximal promoter of *ATP2A3* gene (Contreras-Leal et al., 2016; Flores-Peredo et al., 2016). Also, NaB and TSA increase Sp1, Sp3, and Klf4 levels at the proximal promoter of *ATP2A3* gene, suggesting that these transcription factors have a role in SERCA3 mRNA expression (Flores-Peredo et al., 2016). Despite this evidence, the molecular mechanism of SERCA3 mRNA upregulation by NaB and TSA is not fully understood. This study was aimed to determine whether NaB or TSA modulate SERCA3 mRNA expression in rat hepatocellular carcinoma cells and whether these HDACi modulate acetylation of specific lysines (K9 and K27) of histone 3 in two regions of the *ATP2A3* proximal promoter. Moreover, we explored levels of Sp1 and Sp3 transcription factors, as well as p300, a transcription co-activator and histone acetyltransferase (HAT), at the *ATP2A3* promoter.

## 2. Materials and methods

### 2.1. Analysis of human ATP2A2 and ATP2A3 expression from Oncomine and Kaplan-Meier plotter databases

With the intention to compare *ATP2A2* and *ATP2A3* expression levels between normal liver tissue and hepatocellular carcinoma, we accessed the Oncomine database (OncomineTM Research Edition, 2018), and performed an analysis using The Cancer Genome Atlas (TCGA) liver cancer dataset, including 97 samples of hepatocellular carcinoma, 59 samples of normal liver and 56 samples of normal blood; as well as the Roessler liver 2 dataset (Roessler et al., 2010), that includes 225 hepatocellular carcinoma samples and 220 normal liver samples. To show the association of *ATP2A2* and *ATP2A3* expression with the overall survival of liver cancer patients, a Kaplan-Meier survival plot was generated using the Kaplan-Meier plot database of mRNA-seq (KM-plot, 2018). For the analysis of *ATP2A2* gene, the data of 364 patients were divided into low expression group (217 patients) and high expression group (147 patients). Similarly, for *ATP2A3* analysis, data of 364 patients were divided into low expression group (107 patients) and high expression group (257 patients) (Szász et al., 2016).

### 2.2. Cell culture and treatments

Frozen stocks of AS-30D hepatocellular carcinoma cells were kindly provided by Dr. Jaime Mas-Oliva from the Instituto de Fisiología Celular, Universidad Nacional Autónoma de México. Cells were cultured in RPMI medium with high glucose, supplemented with 10% fetal bovine serum, 0.5% penicillin-streptomycin and 70 mg/L kanamycin (Sigma, St. Louis, MO, USA) at 37 °C in a humidified 5% $CO_2$/95% air atmosphere incubator. For the treatments with histone deacetylase inhibitors, sodium butyrate (NaB) or trichostatin A (TSA), $0.3 \times 10^6$ cells were seeded in 6-well plates. After two days in culture, the medium was changed and replaced with fresh medium with NaB 1, 2, or 3 mM; TSA 50 or 100 nM Cells were harvest after 12, 24, 48 or 72 h of treatment and processed for RNA isolation. The medium containing the indicated inhibitors was replaced every 24 h.

### 2.3. RNA isolation, reverse transcription, and real-time RT-PCR

RNA isolation, reverse transcription, and real-time RT-PCR were performed as previously described (Contreras-Leal et al., 2016). Briefly, AS-30D cells were washed twice with cold PBS, then 1 ml of Trisure reagent was added to each well, whereas liver tissue samples (50–100 mg) were obtained from healthy adult rats and homogenized with 1 ml of Trisure reagent. Total RNA was isolated according to the instructions of the manufacturer (Bioline, Taunton, MA, USA). The RNA concentration was determined with a Nanodrop One (Thermo Scientific, Waltham, MA, USA), and RNA integrity was assessed by 1% formaldehyde-agarose gel.

Briefly, two μg of total RNA were reverse transcribed using 200 U of M-MLV reverse transcriptase in a final volume of 20 μl according to the specifications of the manufacturer (Invitrogen, Thermo Scientific), and then diluted to 40 μl with nuclease-free water. Real-time RT-PCR reactions were performed in triplicate with 1 μl of cDNA (equivalent to 50 ng of the initial RNA), 6 μl of 2x SensiFast SYBRGreen qPCR reaction (Bioline), 5 pmoles of primers for rat SERCA2b or panSERCA3 (Table S1), and water up to 12 μl. PCR conditions were 2 min at 95 °C, 40 cycles of 10 s at 95 °C and 60 s at 60 °C, with a final dissociation analysis, in a 7500 thermal cycler (Applied Biosystems, Foster City, CA). Reactions with β-actin were used for data normalization. PCR efficiencies were determined with the LinReg program, and relative mRNA expression was calculated using the model described by (Pfaffl, 2001).

### 2.4. Histone acetyltransferase activity

HAT activity assay was performed with the HAT Activity Colorimetric Assay Kit (Biovision, CA, USA), according to the manufacturer´s instructions. Briefly, 50 μg of nuclear extracts from control or AS-30D cells treated with NaB or TSA were incubated with HAT substrate at 37 °C. Absorbance was measured at 440 nm in a Wallac Victor$^2$ Luminometer (Perkin Elmer, Waltham, MA).

### 2.5. Chromatin immunoprecipitation

AS-30D cells treated with 3 mM NaB or 100 nm TSA for 24 h were crosslinked with 1% formaldehyde for 20 min. Crosslinking reaction was stopped with 1.25 M glycine, and cells were washed twice with PBS containing 1 mM PMSF, then lysed with lysis buffer [Tris HCl 50 mM pH 8.0, EDTA 10 mM, SDS 1%, and Fast Protease Inhibitor Cocktail (Sigma)]. Chromatin sonication was performed in a Biorruptor Pico (Diagenode, NJ, USA), samples were exposed to 4 sonication cycles. Protein concentration was determined in each sample, and 1 mg of the sonicated chromatin was incubated overnight at 4 °C with 2 μg of specific antibodies for H3K9Ac (Abcam ab4441), H3K27Ac (Abcam ab4729), Sp1 (Santa Cruz sc-59X), Sp3 (Santa Cruz sc-13018X), or p300 (Santa Cruz sc-32244). Immunoprecipitation was performed with One-Day ChIP kit (Diagenode), according to the manufacturer's instructions. Real-time PCR reactions were performed with specific primers (Table S1) for *ATP2A3* gene promoter, spanning two regions -314 to -154 and -164 to +6 (Figure S1). Real-time PCR reactions were performed in triplicate as described above, except that 0.5% DMSO was added to the mix. Data are shown as percent of the input signal.

### 2.6. Spectrofluorometric determination of $Ca^{2+}$

AS-30D cells treated with 3 mM NaB or 100 nM TSA for 24 h were washed twice with PBS, they were loaded with 5 μM FURA2-AM (Thermo Scientific) in Krebs buffer (120 mM NaCl, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 4.75 mM KCl, 10 mM glucose, 20 mM HEPES, 1.27 mM $CaCl_2$, 0.05% BSA, and 0.001% pluronic acid, pH 7.4) for 45 min at 37 °C. Fluorescence was measured at 340 nm excitation, and light emission was recorded at 510 nm in an Aminco Bowman Series 2 Luminescence Spectrometer (Aminco Bowman, Rochester, NY, USA). Thapsigargin (50 μM) was used to measure $Ca^{2+}$ release from the endoplasmic reticulum (ER).

### 2.7. Statistical analysis

Statistical analysis was performed with one-way ANOVA and Tukey posthoc test in STATISTICA 7.0 software (TIBCO, CA, USA). Results are presented as mean ± standard deviation (SD), a p value ≤ 0.05 was considered statistically significant.

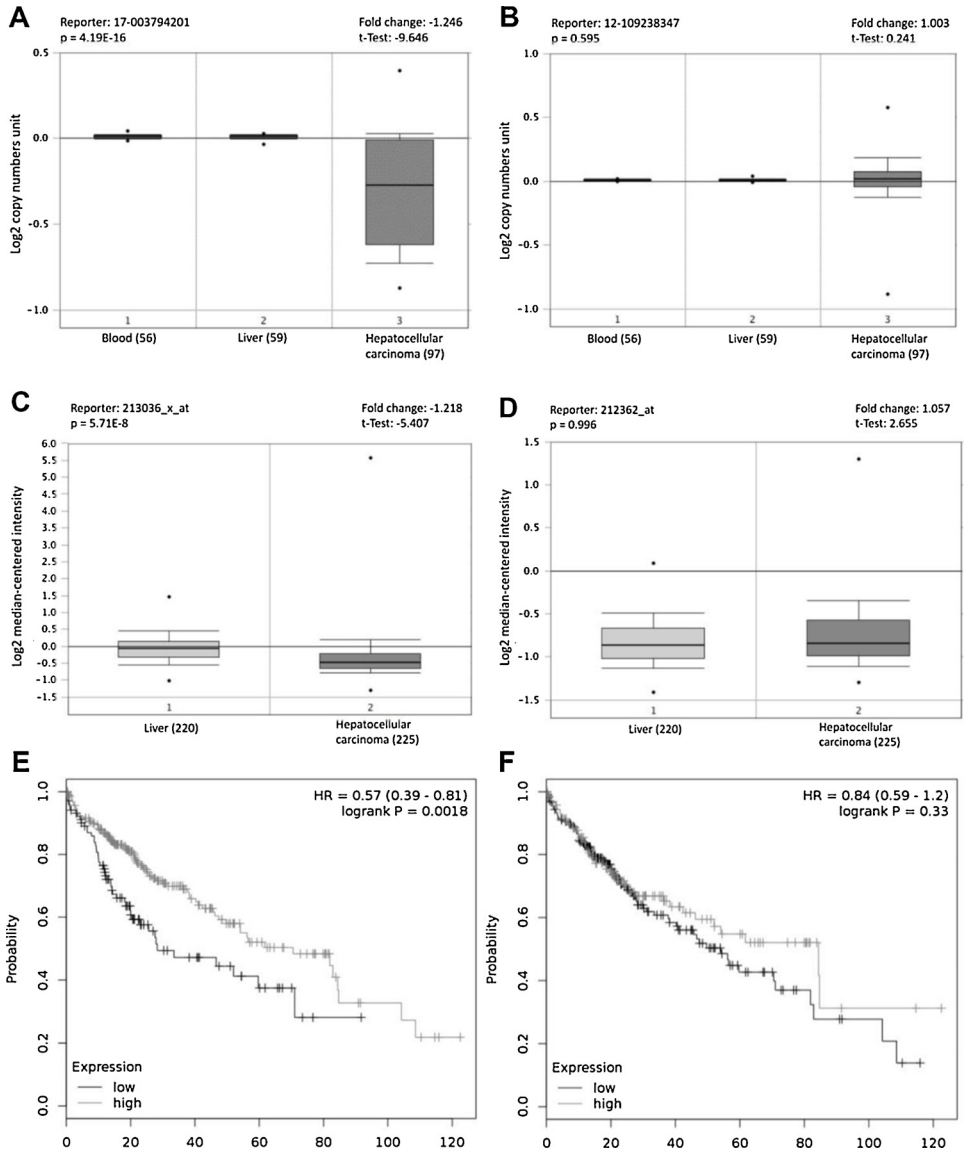


**Fig. 1.** SERCA3 mRNA decreases in human hepatocellular carcinoma samples and its high expression correlates with longer survival of liver cancer patients. SERCA3 mRNA (A and C) and SERCA2 mRNA (B and D) expression were analyzed using the Oncomine database. Kaplan-Meier overall survival plot from liver cancer patients with high and low expression of SERCA3 (E) and SERCA2 (F).

## 3. Results

### *3.1. SERCA3 mRNA expression is decreased in human hepatocellular carcinoma, and its low expression correlates with poor survival*

Analysis of liver cancer datasets for *ATP2A2* (SERCA2) and *ATP2A3* (SERCA3) shows a reduction of SERCA3 mRNA abundance in hepatocellular carcinoma samples compared to normal liver tissue samples (Fig. 1A and C). In contrast, SERCA2 mRNA relative expression displayed similar values between hepatocellular carcinoma and normal liver samples (Fig. 1B and D). In addition, analysis of TCGA liver cancer cohort samples according to their Ishak fibrosis stage or the Child-Pugh score showed that neither SERCA2 nor SERCA3 mRNA expression has significant difference among different liver damage stages (Fig. S2). However, SERCA3 mRNA expression is decreased in hepatocellular carcinoma samples, compared to non-tumor cirrhotic tissue of the same patients in two additional datasets (GEO GSE63898 and GSE17548) (Fig. S3A and S3C), whereas SERCA2 mRNA expression is increased (Fig. S3B and S3D).

Overall survival analysis of hepatocellular carcinoma patients with high and low SERCA2 and SERCA3 mRNA expression showed that patients with high *ATP2A3* expression survived longer, with a median of 70.5 months, compared to patients with low *ATP2A3* expression, with a median of 28.3 months, $p = 0.0018$ (Fig. 1E). Also, HCC patients with high *ATP2A3* expression survive longer, independently of alcohol consumption or hepatitis virus infection (Fig S4). In contrast, patients with high or low *ATP2A2* expression showed no significant difference in overall survival, with a median of 84.4 and 54.1 months, respectively, $p = 0.33$ (Fig. 1F and S4).

### *3.2. SERCA2b and SERCA3 mRNA expression is downregulated in rat hepatocellular carcinoma AS-30D cells*

We quantified the relative mRNA expression of SERCA2b and SERCA3 in rat hepatocellular carcinoma AS-30D cells and control rat liver tissue. We found a reduction of SERCA2b (Fig. 2A) and SERCA3 (Fig. 2B) mRNA levels in AS-30D cells, compared to control rat liver ($0.53 \pm 0.2$ and $0.13 \pm 0.05$, respectively, $p < 0.001$).

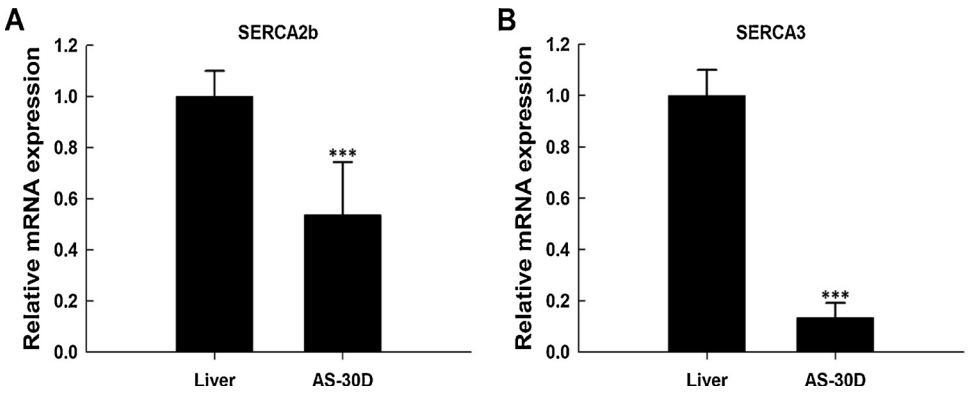


**Fig. 2.** Downregulated SERCA2b and SERCA3 mRNA expression in rat hepatocellular carcinoma AS-30D cells. SERCA2b (A) and SERCA3 (B) mRNA expression were analyzed by qRT-PCR in AS-30D cells and compared to normal rat liver. Data are presented as mean ± SD of eight independent experiments performed in triplicate, ***p < 0.001 compared to control.

### 3.3. NaB and TSA increase SERCA3 mRNA expression in AS-30D cells

Once established that both SERCA2b and SERCA3 mRNA were downregulated in AS-30D cells, we further investigate whether NaB and TSA regulate SERCA2b and SERCA3 mRNA expression. To achieve this, AS-30D cells were treated with different concentrations of NaB or TSA for 48 h. NaB increased SERCA3 mRNA expression in a dose-dependent manner to 3.01 ± 1.05, 5.7 ± 1.6 ($p < 0.01$), and 14.3 ± 4.5 ($p < 0.001$) with 1, 2, and 3 mM NaB, respectively (Fig. 3A). Cells treated with 3 mM NaB also showed a significant difference to those treated with 1 and 2 mM ($p < 0.001$). In addition, 100 nM TSA upregulates SERCA3 mRNA to 5.1 ± 0.1 above control cells ($p < 0.001$), whereas TSA 50 nM shows no difference (Fig. 3B). Cells treated with 100 nM TSA displayed a significant difference to those treated with 50 nM TSA ($p < 0.001$). In contrast, NaB or TSA treatment shows no induction of SERCA2b mRNA expression compared to control cells (Fig. 3C and D).

We performed a time course treatment of AS-30D cells during 12–72 h, to evaluate whether the effect of 3 mM NaB or 100 nM TSA on SERCA3 expression was time dependent. Results show that NaB and TSA increase SERCA3 mRNA expression at 12 h of treatment to 22.3 ± 1.6 and 7.09 ± 1.8 fold, respectively ($p < 0.001$) (Fig. 4). Treatment of AS-30D cells for 24 h increased SECA3 mRNA expression to 24.9 ± 7.17 and 5.9 ± 1.8 fold above control cells ($p < 0.001$) with NaB and TSA, respectively. However, no statistical difference was found between cells treated during 12 or 24 h. Moreover, treatment for 48 h or 72 h shows a lower induction of SERCA3 mRNA to NaB and TSA, compared to 12 h and 24 h (12.1 ± 1.7, $p < 0.001$ and 2.5 ± 0.43, $p = 0.07$, respectively) (Fig. 4).

### 3.4. NaB and TSA increase H3K9 and H3K27 acetylation at the proximal promoter of the ATP2A3 gene

To explore if SERCA3 mRNA upregulation by NaB and TSA was associated with changes on histone acetylation of the *ATP2A3* gene promoter, we first performed a HAT activity assay with nuclear extracts from AS-30D cells treated with 3 mM NaB or 100 nM TSA for 24 h. Both NaB and TSA increase HAT activity to 140% ± 10.1 and 148% ± 8.6, $p < 0.05$, respectively, compared to untreated cells (Fig. 5A). Next, we evaluated by ChIP assays whether the increased HAT activity modulates chromatin acetylation at the *ATP2A3* promoter, using specific antibodies to H3K9ac and H3K27ac, followed by real-time PCR reactions with primers covering two regions of the *ATP2A3* promoter, -314 to -154 and -164 to +6 (Fig. S1). Compared to control cells, we found a

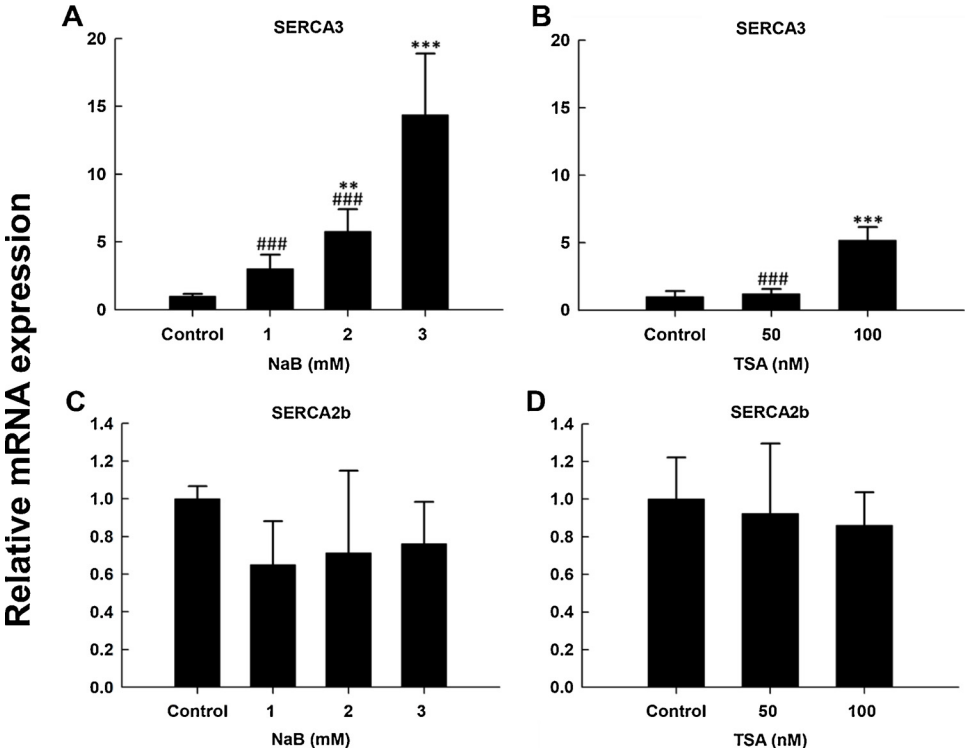


**Fig. 3.** NaB and TSA treatment increase SERCA3 mRNA expression in AS-30D cells, whereas SERCA2b mRNA expression shows no change. AS-30D cells were treated for 48 h with the indicated concentrations of NaB (A and C) or TSA (B and D) and mRNA levels were measured by qRT-PCR. Data are presented as mean ± SD of three independent experiments performed in triplicate. **p < 0.01 and ***p < 0.001 indicate significance compared to control, ###p < 0.001 indicate significance compared with 3 mM NaB in panel A and TSA 100 nM in panel B.

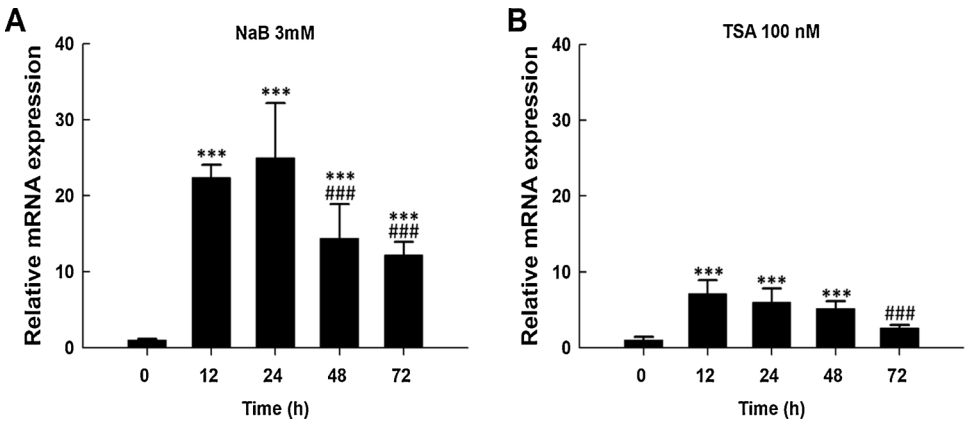


**Fig. 4.** NaB and TSA up-regulate SERCA3 mRNA expression in a time-dependent manner in AS-30D cells. AS-30D cells were treated with 3 mM NaB (A) or 100 nM TSA (B) for 12, 24, 48, and 72 h. Data are presented as mean ± SD of three independent experiments performed in triplicate, ***p < 0.001 indicate significance compared to control, ###p < 0.001 indicate significance compared to 24 h in panel A and panel B.

significant increase of H3K9ac at both regions of the *ATP2A3* promoter in cells treated with NaB (p < 0.001) (Fig. 5B and C), whereas TSA increased H3K9Ac only at the -314 to -154 promoter region, p < 0.01 (Fig. 5B). Similarly, an increase of H3K27Ac was found in cells treated with NaB and TSA at both promoter regions (Fig. 5D and E). A larger effect was observed with NaB than TSA, and H3K27Ac also showed higher levels than H3K9Ac (Fig. 5).

### 3.5. NaB increases Sp1 and Sp3 binding at the proximal promoter of *ATP2A3* gene

It has been shown that Sp1 and Sp3 transcription factors regulate SERCA3 mRNA expression. Particularly, (Hadri et al., 2002) showed that Sp1 binding between -97 to +153 of *ATP2A3* gene was essential for its basal transcription, and KATO-III cells treated with NaB or TSA increase Sp1 and Sp3 recruitment to the *ATP2A3* gene proximal promoter (-162 to +39) (Flores-Peredo et al., 2016). *In silico* analysis showed the presence of one Sp1/Sp3 putative DNA binding site at -314 to -154 region, as well as two Sp1/Sp3, one Sp1, and one Sp3 putative DNA binding sites at -164 to +6 of the rat *ATP2A3* promoter (Fig. S1). Therefore, we evaluate whether Sp1 or Sp3 binding increased at the *ATP2A3* gene promoter after NaB or TSA treatment. ChIP results show an increase of Sp1 binding to the -164 to +6 region in cells treated with NaB, p < 0.001 (Fig. 6B); whereas Sp3 binding increased at the -314 to -154 region with the NaB treatment (p < 0.05, Fig. 6C). TSA treated cells display no difference in Sp1 or Sp3 binding to the two promoter regions compared with control cells.

### 3.6. p300 is important for basal and TSA-mediated SERCA3 mRNA expression in AS-30D cells

We further investigate which other mechanisms could be involved in the up-regulation of SERCA3 mRNA mediated by NaB or TSA. p300, a well-known HAT, was recently associated with increased acetylation of H3K9 and H3K27 (Qiao et al., 2015; Jin et al., 2011; Li et al., 2013; Hilton et al., 2015). Thus, we tested the binding of p300 to the *ATP2A3* promoter after NaB or TSA treatment. Results showed a marked increase of p300 recruitment to both *ATP2A3* promoter regions after TSA treatment (p < 0.001), compared to control cells; whereas NaB treated cells show no difference compared to control (Fig. 7A and B). We next examine the role of p300 in TSA-mediated SERCA3 mRNA expression using the specific p300 inhibitor, C646 (Bowers et al., 2010). Results of real-time PCR show that C646 decreased the basal SERCA3 mRNA expression compared to untreated cells (Fig. 7C). Moreover, TSA-mediated SERCA3 mRNA up-regulation was abolished by C646 (p < 0.001). This result is the first evidence showing the role of p300 in the regulation of SERCA3 mRNA expression.

### 3.7. Inhibition of p300 decrease acetylation of H3K9 at ATP2A3 gene promoter in TSA treated cells

Once established that p300 is involved in basal and TSA-mediated up-regulation of SERCA3 mRNA, we investigated whether p300 inhibition by C646 has any effect on H3K9 and H3K27 acetylation observed in TSA treated cells (Fig. 5). ChIP results from AS-30D cells treated with TSA or TSA + C646 showed a significative reduction of H3K9ac at the two promoter regions when p300 was inhibited,

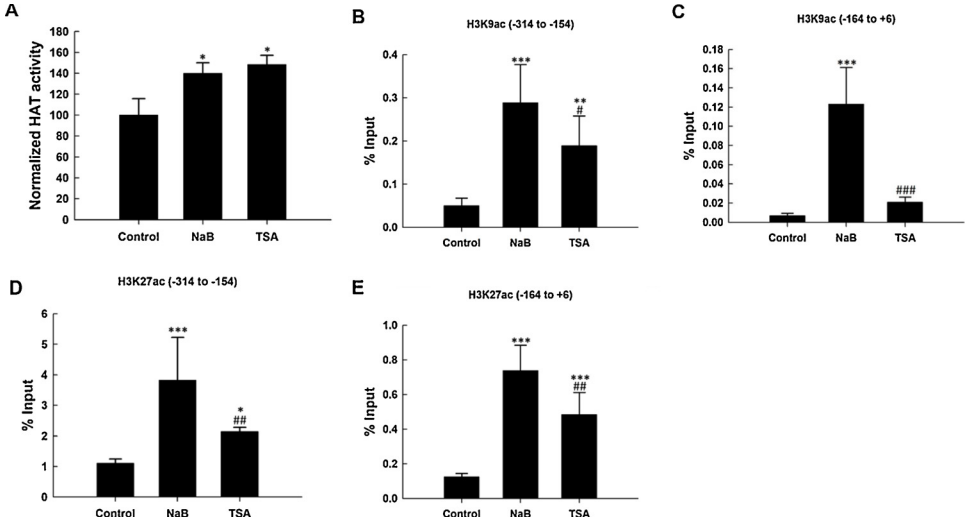


**Fig. 5.** NaB and TSA treatment modulates H3 acetylation at the proximal promoter region of *ATP2A3* gene. AS-30D cells were treated with 3 mM NaB or 100 nM TSA for 24 h; then chromatin was processed for ChIP assays using antibodies specific to H3K9ac or H3K27ac, followed by real time-PCR. HAT activity assay performed in nuclear extracts of AS-30D cells treated with NaB or TSA (A). ChIP results of H3K9ac in two regions of the proximal *ATP2A3* promoter (B and C), ChIP results of H3K27ac in the same promoter regions (D and E). Data are presented as mean ± SD of three independent experiments performed in triplicate. *p < 0.05, **p < 0.01, and ***p < 0.001 indicate significance compared to control, #p < 0.05, ##p < 0.01, ###p < 0.001 indicate significance compared to NaB in panels B, C, D, and E, respectively.

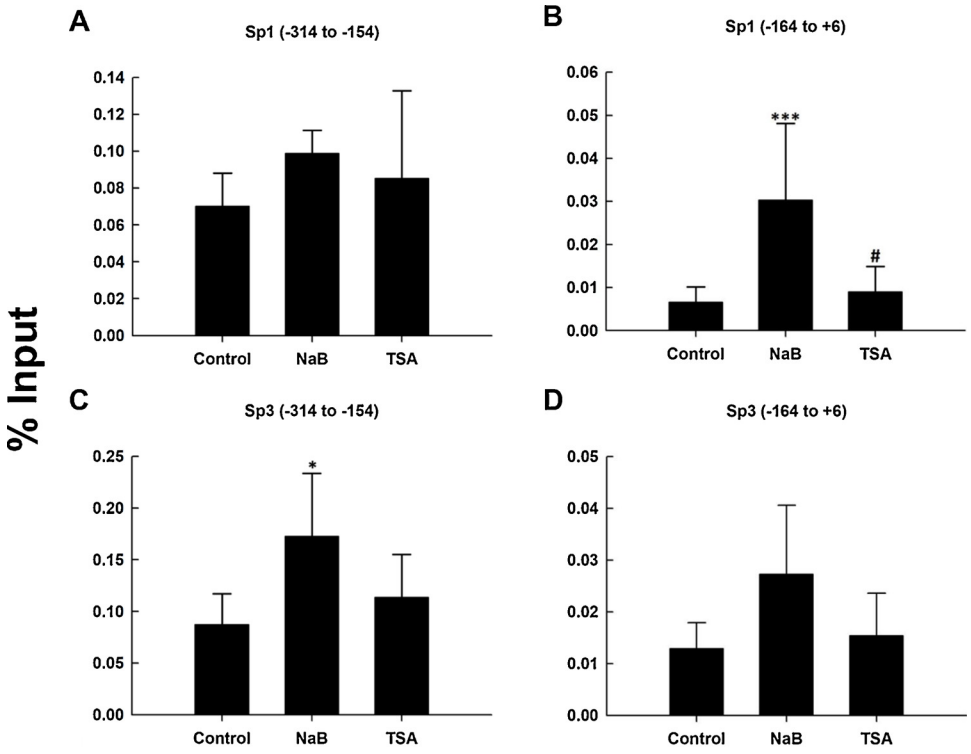


**Fig. 6.** NaB treatment modulates Sp1 and Sp3 recruitment to the proximal promoter of *ATP2A3* gene. AS-30D cells were treated with 3 mM NaB or 100 nM TSA for 24 h; then chromatin was processed for ChIP assays using antibodies specific to Sp1 or Sp3, followed by real time-PCR. ChIP results of Sp1 (A and B) and Sp3 (C and D) are shown. Data are presented as mean ± SD of three independent experiments performed in triplicate. *p < 0.05, and ***p < 0.001 indicate significance compared to control, #p < 0.05 indicate significance compared to NaB in panel B.

compared to TSA treated cells, p < 0.001 (Fig. 7D and E). In contrast, no significant effect was found on H3K27ac at both promoter regions analyzed (Fig. S5A and S5B).

### 3.8. NaB and TSA modulate intracellular $Ca^{2+}$ concentration in AS-30D cells

To determine the effects of NaB and TSA on the intracellular $Ca^{2+}$ handling, we measured the basal cytosolic $Ca^{2+}$ and thapsigargin-induced $Ca^{2+}$ release from the endoplasmic reticulum in AS-30D cells. A significant increase in basal cytosolic $Ca^{2+}$ was found in NaB and TSA treated cells (74.9 ± 1.9 nM and 89.8 ± 10.2 nM, respectively) compared to untreated cells (37.7 ± 3.9 nM), p < 0.001, (Fig. 8). Moreover, TSA treated cells displayed a significant increase in thapsigargin-induced $Ca^{2+}$ release (109.2 ± 17 nM, p < 0.001, Fig. 8C), compared to control cells (90.2 ± 6.7 nM, Fig. 8A). In contrast, NaB treated cells showed no significant difference in thapsigargin-induced $Ca^{2+}$ release (95.8 ± 16.2 nM, Fig. 8B).

## 4. Discussion

The importance of SERCA2 and SERCA3 in hepatocellular carcinoma has not been established. Analysis of liver cancer datasets in Oncomine database showed lower *ATP2A3* expression levels in human hepatocellular carcinoma samples compared to normal liver tissue, whereas *ATP2A2* expression remains similar to normal liver. Interestingly, no difference in SERCA2 or SERCA3 mRNA expression was found among different stages of Ishak fibrosis stage or Child-Pug score of liver function in TCGA liver cancer samples. However, SERCA3 mRNA expression is decreased and SERCA2 mRNA expression is increased in HCC compared to non-tumor cirrhotic tissue of the same patients, in two additional datasets, suggesting that alteration of

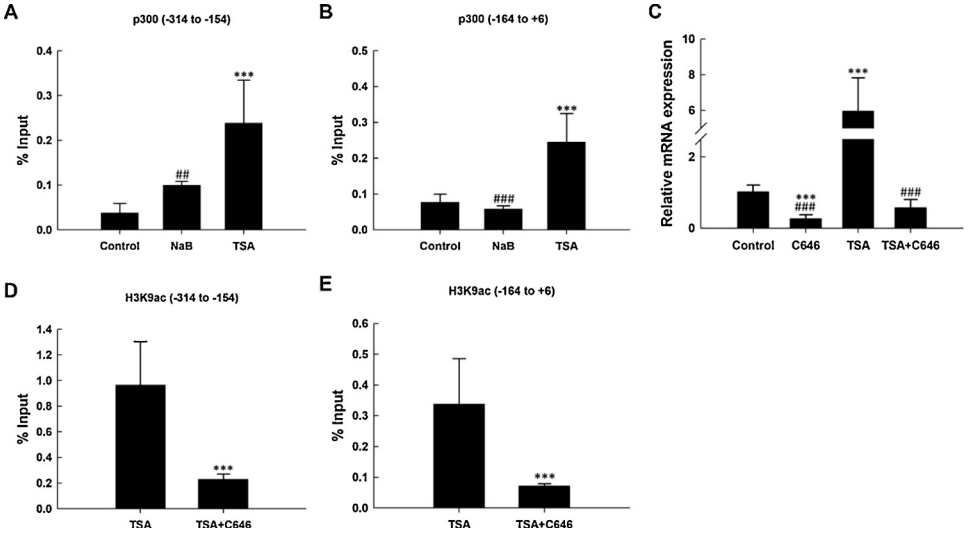


**Fig. 7.** p300 is involved in *ATP2A3* basal transcription and TSA-mediated upregulation. AS-30D cells were treated with 3 mM NaB, 100 nM TSA or TSA + C646 (100 μM) for 24 h, then chromatin was processed for ChIP assays using a specific antibody to p300 (A and B). AS-30D cells were treated with 100 nM TSA, 100 μM C646, or both for 24 h, then RNA was isolated and used for qRT-PCR experiments (C). ChIP results obtained for H3K9ac in TSA and cells treated with TSA + C646 (D and E). Data are presented as mean ± SD of three independent experiments performed in triplicate. ***p < 0.001, indicate significance compared to control and in panels D and E indicate significance compared to TSA, ##p < 0.01 indicate significance compared to TSA in panel A, and ###p < 0.001 indicate significance compared to TSA in panel B and C.

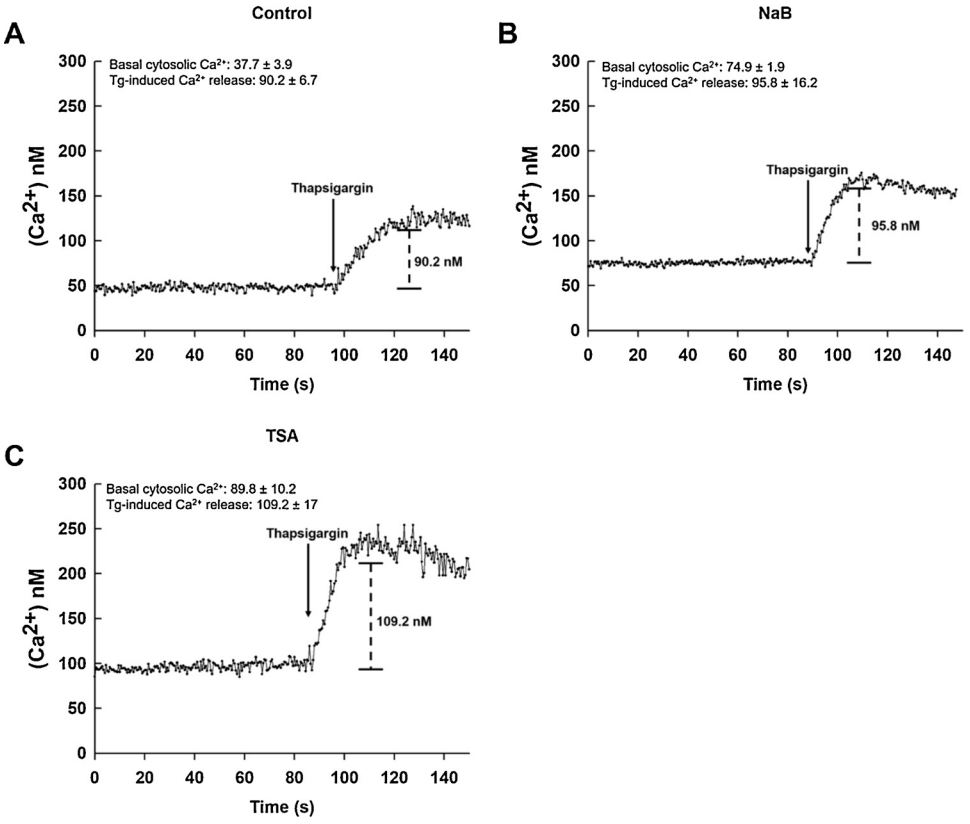


**Fig. 8.** Intracellular $Ca^{2+}$ levels in control and AS-30D cells treated with NaB or TSA. To determine basal cytosolic $Ca^{2+}$, control and AS-30D cells treated with NaB or TSA for 24 h were incubated with the $Ca^{2+}$ indicator FURA 2-AM, once the signal was stabilized, endoplasmic reticulum $Ca^{2+}$ content was determined by adding thapsigargin (Tg), a specific SERCA inhibitor. Data are presented as mean ± SD of three independent experiments performed in triplicate.

SERCA2 and SERCA3 expression could take place during the transition of cirrhosis to HCC. Moreover, high expression of *ATP2A3* gene correlates with longer survival in patients with liver cancer, whereas no significant difference was found in patients' survival with high and low expression of the *ATP2A2* gene and this correlation was independent of alcohol consumption and hepatitis virus infection. These results suggest that *ATP2A3* (SERCA3) gene expression could have a role in liver cancer and patients' survival.

We also found a marked reduction of SERCA2b and SERCA3 mRNA expression in rat AS-30D cells compared to normal rat liver. Downregulation of SERCA2 and SERCA3 expression has been found in several types of cancer such as oral, breast, lung, colon, gastric, and choroid plexus (Endo et al., 2004; Brouland et al., 2005; Gélébart et al., 2002; Papp and Brouland, 2011; Arbabian et al., 2013; Ait-Ghezali et al., 2014). Moreover, an association between decreased SERCA3 expression and tumor progression has been found in breast, gastric, and choroid plexus cancer samples (Gélébart et al., 2002; Papp and Brouland, 2011; Ait-Ghezali et al., 2014).

We and others have previously shown that NaB or TSA up-regulate SERCA3 mRNA and protein expression in breast and gastric cancer cell lines (Gélébart et al., 2002; Contreras-Leal et al., 2016; Flores-Peredo et al., 2016). Here we have shown that NaB and TSA induced SERCA3 mRNA expression, in a dose- and time-dependent manner, in AS-30D cells, whereas SERCA2 mRNA levels do not change with these HDACi. Higher levels of SERCA3 mRNA were found with 3 mM NaB and 100 nM TSA after 12 or 24 h of treatment, whereas 48 and 72 h of treatment displayed a lower induction of SERCA3 mRNA. These findings are different from those reported by (Gélébart et al., 2002) who found high SERCA3 protein expression in gastric and colon cancer cells after 72 to 96 h of treatment with 3 mM NaB. Transient effect of HDACi on gene expression has been previously observed. NaB increases gastrin mRNA in a time-dependent manner with a higher effect at 48 h of treatment (Simon et al., 1997). On the other hand, p21 and BIM mRNA expression displayed a transient increase in cells treated with 200 nM TSA (Moore et al., 2004).

We found an increase in HAT activity in response to NaB and TSA treatments, as well as an increment of H3K9 and H3K27 acetylation at two *ATP2A3* promoter regions. NaB treatment increases H3K9 and H3K27 acetylation at -314 to -154 and -162 to +6, whereas TSA increased H3K9 acetylation only at -314 to -154, and H3K27 acetylation at both regions. The differential effect of NaB and TSA could be the explanation for the different SERCA3 mRNA levels induced by these HDACi. Previous reports indicate an increment of H3K9ac at -162 to +39 of the *ATP2A3* promoter region in breast and gastric cancer cell lines after treatment with NaB or TSA (Contreras-Leal et al., 2016; Flores-Peredo et al., 2016). On the other hand, Meneses-Morales et al. saw a marked increase of H3K27ac at -265 to -135 of the *ATP2A3* promoter region in KATO-III cells treated with NaB (Meneses-Morales et al., 2019). A differential promoter acetylation pattern after stimuli has been found in other promoters. Romidepsin differentially acetylates histone H3 at *ABCG2* proximal and distal promoter in S1 colon cancer cell line (To et al., 2008). (Roy et al., 2014) showed a differential acetylation pattern of H3K9, H3K14, and H3K27 between -610 to +307 of the *OsDREB1b* gene promoter after stress stimuli.

Another reported mechanism of NaB and TSA on gene expression is through increased occupancy of transcription factors such as Sp1 and Sp3 in gene promoters (Walker et al., 2001; Kuan et al., 2016). Our results showed that only NaB increases binding of Sp1 and Sp3 at the *ATP2A3* gene promoter. Notably, Sp1 levels increased at -164 to +6, whereas Sp3 levels increased at -314 to -154. (Hadri et al., 2002) showed that Sp1 is critical for the basal transcription of the mouse SERCA3 gene. Moreover, the occupancy of Sp1 and Sp3 at the *ATP2A3* gene promoter increased in KATO-III cells treated with NaB or TSA (Flores-Peredo et al., 2016). Together these results suggest that Sp1 and Sp3 are important for SERCA3 gene expression and this mechanism is conserved at least in three different species (human, mouse, and rat). Our data suggest that Sp1 and Sp3 are not involved in the *ATP2A3* mRNA upregulation by TSA; therefore, we explored which other mechanisms could be involved in TSA mediated SERCA3 upregulation and increased H3K9ac and H3K27ac to the promoter.

ChIP results showed increased p300 occupancy to both regions of the *ATP2A3* gene promoter analyzed in AS-30D cells treated with TSA. Moreover, when we chemically inhibited p300 with C646, a specific inhibitor (Bowers et al., 2010), the effect of TSA on *ATP2A3* mRNA expression was abolished entirely. Added to this, AS-30D cells treated with C646 displayed a 70% decrease in basal SERCA3 mRNA expression compared to untreated cells. These results suggest that p300 is important in TSA-mediated SERCA3 mRNA upregulation and basal *ATP2A3* transcription in AS-30D cells. In contrast, NaB treated cells showed no difference in p300 occupancy at the *ATP2A3* promoter compared to untreated cells. Our results are in agreement with those reported by (Li et al., 2013), who found that C646 reduced *11β-HSD2* gene expression due to the crucial role of p300 in this process. Recent evidence showed that p300 is important in several liver functions. (Breaux et al., 2015) reported that p300 is involved in normal mouse liver development, regulating hepatocyte proliferation, as well as C/EBPα and p53 protein expression.

We further investigate the role of p300 in the acetylation of H3K9 and H3K27 at *ATP2A3* gene promoter. TSA-mediated acetylation of H3K9 was reduced in AS-30D cells treated with TSA + C646 compared to cells treated with TSA alone. In contrast, no significant effect was found in H3K27 acetylation. These results suggest that acetylation of H3K9 at the *ATP2A3* gene proximal promoter in TSA-treated cells is mediated by p300 and relevant for *ATP2A3* up-regulation. Our results are in accordance with those reported by (Li et al., 2013) and (Crump et al., 2011), who found that inhibition of p300 by C646 significantly reduced acetylation of H3K9 at the promoters of *11β-HSD2*, *c-fos,* and *c-jun* genes. TSA-treated cells displayed enrichment of H3K9ac and H3K27ac; however, C646 completely abolished TSA-mediated up-regulation of SERCA3 mRNA and TSA-mediated increase of H3K9ac, with no effect on H3K27ac. H3K27ac was associated with the recruitment of RNA Pol II, whereas H3K9ac was associated with the transition of transcription initiation to elongation (Jin et al., 2011; Stasevich et al., 2014; Gates et al., 2017). These distinct roles of H3K9ac and H3K27ac in gene regulation could explain the sustained H3K27ac in TSA + C646 treated cells despite the marked reduction in SERCA3 mRNA expression. Thus, we propose that together H3K9ac and H3K27ac are important for TSA-mediated SERCA3 up-regulation.

Many liver functions, such as bile secretion, glucose metabolism, cell proliferation, and apoptosis, are regulated by the increase and decrease of intracellular $Ca^{2+}$ concentrations (Amaya and Nathanson, 2013). We explored whether NaB and TSA treatments modulate intracellular $Ca^{2+}$ handling in AS-30D cells. We found a significant increase of basal cytosolic $Ca^{2+}$ concentration in NaB and TSA treated cells compared to untreated cells, whereas only TSA significantly increased the $Ca^{2+}$ released from the endoplasmic reticulum. (Gélébart et al., 2002) and (Flores-Peredo et al., 2016) found an increase of the basal cytosolic $Ca^{2+}$ and a reduction of the $Ca^{2+}$ released from the endoplasmic reticulum in KATO-III cells treated with NaB or TSA. Nevertheless, it is difficult to attribute the difference of the intracellular $Ca^{2+}$ handling to SERCA3 expression only, due to the presence of other $Ca^{2+}$ handling proteins and buffering systems.

In conclusion, SERCA3 expression, a major modulator of $Ca^{2+}$ homeostasis, is decreased in human HCC samples and the rat HCC AS-30D cell line, compared to normal liver, and high levels of SERCA3 mRNA correlate with longer HCC patient's survival. Additionally, we found a clear decrease in SERCA3 expression in HCC samples compared to cirrhotic liver. In AS-30D cells, NaB and TSA upregulate SERCA3 mRNA expression by increasing H3K9 and H3K27 acetylation and differential recruitment of transcriptional machinery proteins into the *ATP2A3* gene promoter. Notably, we describe for the first time the role of p300 in SERCA3 expression and acetylation of H3K9 at *ATP2A3* gene proximal promoter in AS-30D cells. Together, these results suggest that SERCA3 could have a role in HCC, and give new insights into the molecular mechanism of NaB and TSA-mediated SERCA3 expression. The understanding of epigenetic changes in HCC could contribute to the development of new therapies, and our data suggest that HDACi could be an excellent alternative to reactivate some downregulated genes in HCC, such as *ATP2A3*.

## Funding

The present study was supported by grants from Consejo Nacional de Ciencia y Tecnología, México (grant No. 2015-01-1518 to JS-G), Dirección General de Asuntos del Personal Académico de la Universidad Nacional Autónoma de México (grant PAPIIT No. IN217216 to AZ-H), and AH-O was supported by a scholarship No. 443164 from Consejo Nacional de Ciencia y Tecnología, México.

## Acknowledgments

We thank Laboratorio Nacional de Servicios Experimentales del Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (LaNSE-CINVESTAV-IPN) for their technical assistance with chromatin sonication.

## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biocel.2019.05.014.

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