Trichostatin A alleviates HBx-induced HCC metastasis in metabolic stress through up-regulating SIRT3 expression

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Research

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Abstract

Background

Hepatocellular carcinoma (HCC), with Hepatitis B viurs X protein (HBx) as one of the main etiology, harbors various metabolic phenotypes for distinct nutrient availability inside solid tumors. Trichostatin A is a histone inhibitor, supposed to inhibit cancer development. However, the role of TSA in tumor cells with metabolic stress has received little investigation.

Methods

We built a HBx-overexpression hepatoma cell model with HBx plasmids transfection, and EBSS was applied in nutrient-deprived cell model. Through western blot, Q-PCR and immunoprecipitation, we further explored the molecular mechanism of TSA-related anti-cancer function.

Results

In our study here, we found that TSA inhibited HBx-enhanced metastasis in metabolic stress, and demonstrated that Sirtuin 3 (SIRT3), one of tumor suppressors in HCC, played a role in TSA-related anti-cancer function in metabolic stress, and TSA promoted SIRT3 transcription.

Conclusions

We suggest that TSA alleviated specific subset of HCC, the HBx-induced HCC in metabolic stress, through promoting SIRT3 transcription, suggesting that TSA is a potential drug for HCC treatment.

Background

Environmental nutrient availability is a vital regulator of cancer cell metabolism, resulting in various metabolic phenotypes of tumor cells[1, 2]. The heterogeneity of cancer metabolic phenotype presents challenges for cancer treatments[1]. Among tumor cell populations, some are suffering from metabolic stress as for nutrient shortage, with adaptive metabolic switching to glycolysis or autophagy for survival[3, 4]. For tumor treatment, omission of any tumor cells means cure failure, so an ideal chemoprevention strategy is to target all metabolic phenotypes, including tumor cells in metabolic stress.

Hepatocellular carcinoma (HCC), with hepatitis B virus (HBV) as a main etiology, is a deadly tumor with limited chemoprevention strategies[5, 6]. Hepatitis B virus X protein (HBx), an HBV genome encoding protein, exhibits numbers of effects on promoting HCC progression, including inhibiting hepatoma apoptosis, accelerating epithelial–mesenchymal transition (EMT)[7, 8]. Interestingly, we have reported that HBx played opposing roles on hepatoma apoptosis when in face with distinct nutrient availability[9].

In metabolic stress, HBx promotes hepatoma survival through elevating mitophagy[9]. We also showed that HBx elevated HCC metastasis in nutrient-deprived microenvironment[10]. However, strategies to alleviate enhanced metastasis stimulated by HBx under metabolic stress have received little investigation.

Trichostatin A (TSA), a classical deacetylase inhibitor secreted by streptococcus, is a potential anti-liver cancer drug through stimulating apoptosis, reducing the proliferation, clonogenicity and migratory potential of HCC cells[11–13]. Mechanismly, TSA acts as a histone deacetylase inhibitor (HDACi) to induce hyperacetylation of histone, resulting in enhanced binding of DNA and nuclear transcription factors, modulating mRNA expression of abundant genes[14, 15]. Moreover, a growing number of non-histone proteins are documented to be hyperacetylated targets of TSA[16, 17]. Although several clinical trials of HDACi are undergoing, no unified results are obtained from solid tumors[12]. It's reported that TSA blocks angiogenesis under hypoxic conditions[18]. The diversity of microenvironment in solid tumors may count for the inconsistent trial results. However, the role of TSA in tumor cells with metabolic stress has received little investigation.

In this study, we focus on the effect of TSA on HCC metastasis in metabolic stress, especially for HBx-induced HCC metastasis, and exploring the underlying mechanisms via HBx-overexpressing HCC cell lines, western blotting, quantity PCR, immunoprecipitation.

Methods

Cell culture

Huh7 cells, provided by Cell Bank (Chinese Academy of Sciences), were maintained in DMEM medium (Hyclone) supplemented with 10% fetal bovine serum (Hyclone), adding extra 100× MEM Non-Essential Amino Acids Solution (NEAA, gibco, #11140050) and GlutaMAX (gibco, #35050061). Nicotinamide (United States Sigma Company, #72340) were dissolved with DMEM, and final working concentration that used in the experiment is 25 mM, action time was 16 hr. Trichostatin A (United States Sigma, #V900931), was dissolved with DMSO, prepared with 1000× as mother liquor, and the final working concentration was 5 mM, action time was 16 hr.

Transfection and Lentiviral infection

Plasmids extraction and plasmids transfection were performed as described previously[9]. HBx-expressing plasmids pcDNA3.1-flag- HBx (adw subtype) and pcDNA3.1-vector were obtained as a gift from Professor Lin Xu (School of Basic Medical Sciences, Fujian Medical University). Recombinant lentiviruses containing 3 short hairpin SIRT3 (shSIRT3) sequences, and no-load control non-target short hairpin RNA (NT-shRNA) were purchased from United States Santa cruz company. Steps to construct stabilized shSIRT3-Huh7 cell line were as follows: When Huh7 cells reached about 40% density, a proportionally mixed infection solution (United States Santa cruz, sc-134220) were added and worked on

over 24 hr, transfected cells were then selected with puromycin (United States Santa cruz, #sc-205821) for 1 week to generate stable cells lines.

Quantitative RT-PCR

Total RNA extraction and qRT-PCR were performed as described previously[9]. The primers used were as follows, human SIRT3 for transcript 1+2 forward 5'-AGAGGTTCTTGCTGCATGTG-3', human beta-ACTB forward 5'-CCTGGCACCCAGCACAAT-3'.

Western blot and immunoprecipitation

Total protein extraction and western blot analysis were performed as previously described[9]. Steps of immunoprecipitation assays were as follows: treated cells were resuspended in IP lysis buffer on ice for 5min, and then centrifuged at 14,000 g for 15 min. The supernatants were precleared with protein A/G-coupled agarose (United States Santa cruz, #sc-2003), and subsequently incubated with 2 μg of the indicated antibodies and 20μl protein A/G agarose overnight at 4℃. After washing 5 times with lysis buffer, immunoprecipitates were boiled in 5× loading buffer for western blot analysis as mentioned. Western blot used the following primary antibodies: anti-human SIRT3, anti-acetylated-lysine, anti-human E-cad and anti-rabbit conformational specific IgG from Cell Signaling Technology, anti-human N-cad from BD Company, anti-human beta-actin form Sigma.

Statistical analysis

Data were exhibited as mean values ± standard error (mean ± SEM) of 3 independent experiments. Differences were evaluated by two-tailed Student's t-test. Statistical significance was set at *P < 0.05.

Results

TSA inhibited HBx-enhanced metastasis in metabolic stress.

TSA is supposed to inhibited HCC metastasis[19]*. We confirmed this by western blotting and found that E-cad expression was elevated in Huh7 cells treated with TSA. As we have revealed that HBx specially promoted HCC metastasis in metabolic stress, with EBSS starvation for 3 hr[9], we here explored whether TSA was able to alleviated the enhanced metastasis potential in starvation. Similarly, Huh7 cells was deprived of nutrients with EBSS for 3hr. Huh7 cells were overexpressed with HBx plasmids and with western blotting, we showed that HBx upregulated N-cad expression and synchronously downregulated E-cad expression in Huh7 cells in metabolic stress (Fig. 1). Moreover, we demonstrated that TSA reversed the HBx-induced N-cad, E-cad expression (Fig. 1). These results depicted that TSA inhibited HBx-enhanced metastasis in metabolic stress.

SIRT3 plays a role in TSA-related anti-cancer function in metabolic stress.

SIRT3 is a nutrient sensitive protein [20, 21], and is reported to suppress HCC metastasis[22, 23]. We here explore whether SIRT3 takes a part in TSA-related tumor remission. Huh7 cells were transfected with HBx plasmids and in starvation with EBSS. Western blot illustrating that the expression of SIRT3 was lower in HBx-overexpressing Huh7 cells compared to vector control cells in starvation, while this reduced expression was reversed by TSA administration (Fig. 2). Moreover, we verified that when SIRT3 gene were knocked down by shSIRT3 interference, N-cad and E-cad expression reversed by TSA no longer existed (Fig. 2). These results indicated that SIRT3 is implied in the TSA-related anti-cancer function in metabolic stress.

TSA promotes SIRT3 protein expression.

We then examined the effect of TSA on SIRT3. Huh7 cells were applied with TSA, and western blot showed that SIRT3 protein expression was extremely increased in TSA group, compared to DMSO solvent control group (Fig. 3). In addition, NAM, another deacetylase inhibitor targeting other deacetylase group, only slightly upregulated SIRT3 expression (Fig. 3). These results demonstrated that TSA promoted SIRT3 protein expression.

TSA induces SIRT3 transcriptional expression.

We further analyzed the mechanisms underlying TSA promoting SIRT3 protein expression. TSA is a deacetylase inhibitor, which was confirmed by the increase in acetyl-tubulin levels after TSA administration (Fig. 3). TSA either acts as a histone deacetylase inhibitor to regulates mRNA expression, or as a deacetylase inhibitor targeting post-translational modification[24, 25]. Q-PCR results showed that the SIRT3 mRNA expression in Huh7 cells in metabolic stress was significantly elevated in TSA-treating group, compared to solvent control group (Fig. 4A). Furthermore, with immunoprecipitation, we found that Huh7 cells in starvation with HBx expression displayed hyperacetylation of SIRT3 protein compared to vector-transfected control cells in starvation, which was lowered when in adequate nutrition (Fig. 4B). As we also showed that HBx downregulated SIRT3 protein in Huh7 cells with metabolic stress (as shown in Fig. 2),this founding was consistent with the notion that deacetylated SIRT3 inhibited SIRT3 protein degradation[26]. This result also ruled out that TSA modulated SIRT3 protein amounts through post-translational modification. Collectively, these results demonstrating that TSA upregulated SIRT3 expression through transcriptional modification.

Discussion

There are limited chemotherapy strategies for HCC, partly resulting from intratumor heterogeneity of HCC[27, 28]. The metabolic phenotype of HCC in nutrient stress, characterized with altered survivability and transferability in tumor cells, is one of HCC subgroups urgently requiring effectively new therapy methods. In our study here, we found that TSA alleviated HBx-induced HCC metastasis in metabolic stress, and up-regulated SIRT3 expression was implied in this process. As HBV is a common cause of HCC[29], the specially inhibiting of HBV-induced HCC by TSA, makes TSA a widely applicable drug for HCC. On the other hand, TSA also plays anti-fibrotic role through inhibit differentiation of hepatic stellate

cells[30, 31]. Since most HCC develops from liver fibrosis[32], TSA treatment also advantage in improving tumor microenvironment. Collectively, TSA multiply benefits for HCC treatment.

There are abundant of clinical trials in testing histone acetylase inhibitors for alleviating HCC. However, most of these are fail in solid tumor partially for cytotoxicity and non-specific injury[33]. Since we have emphasized the intratumor heterogeneity with nutrient availability especially for solid tumor, and we showed that TSA took a role in the subgroup with metabolic stress, it's sensible to design combination therapy with TSA to cover all tumor subsets, improving treatment efficacy.

SIRT3 is a newly discovered deacetylase in Sirtuins family, characterizing in modifying mitochondrial protein's acetylation[34, 35]. There are prolific studies identifying SIRT3 as a tumor suppressor in HCC development through mechanisms including regulating mitochondrial metabolism and oxidative stress[22, 36, 37]. Our study here implied that SIRT3 participated in the process of TSA- inhibiting HBx-related HCC, again confirming the tumor suppressive role of SIRT3. TSA and SIRT3 are both deacetylase inhibitors, targeting nuclear histone and mitochondrial protein respectively. We showed that TSA increased the SIRT3 transcription, implying that deacetylases are orchestrated as a network in tumor cells and mutual benefit, which may worth further study.

Conclusions

Our study here demonstrated that TSA alleviated specific subset of HCC, the HBx-induced HCC in metabolic stress, through promoting SIRT3 transcription, suggesting that TSA is a potential drug for HCC treatment.

Abbreviations

HCC
Hepatocellular carcinoma
HBx
Hepatitis B viurs X protein
TSA
Trichostatin A
EBSS
Earle's Balanced Salt Solution
SIRT3
Sirtuin 3
EMT
epithelial–mesenchymal transition
HDACi
histone deacetylase inhibitor
NEAA

Non-Essential Amino Acids Solution
DMSO
dimethyl sulfoxide
Hr
hour
Min
minite
g
relative centrifugal force
mM
mmol/l
P
p value

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Availability of data and materials The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests The authors declare that they have no competing interests.

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Authors' contributions HXY and XGT: writing and draft preparation. HTX, GWY and CZX: methodology and data analysis. ZBY and WXZ: supervision and critical review. The authors read and approved the final manuscript.

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Figures

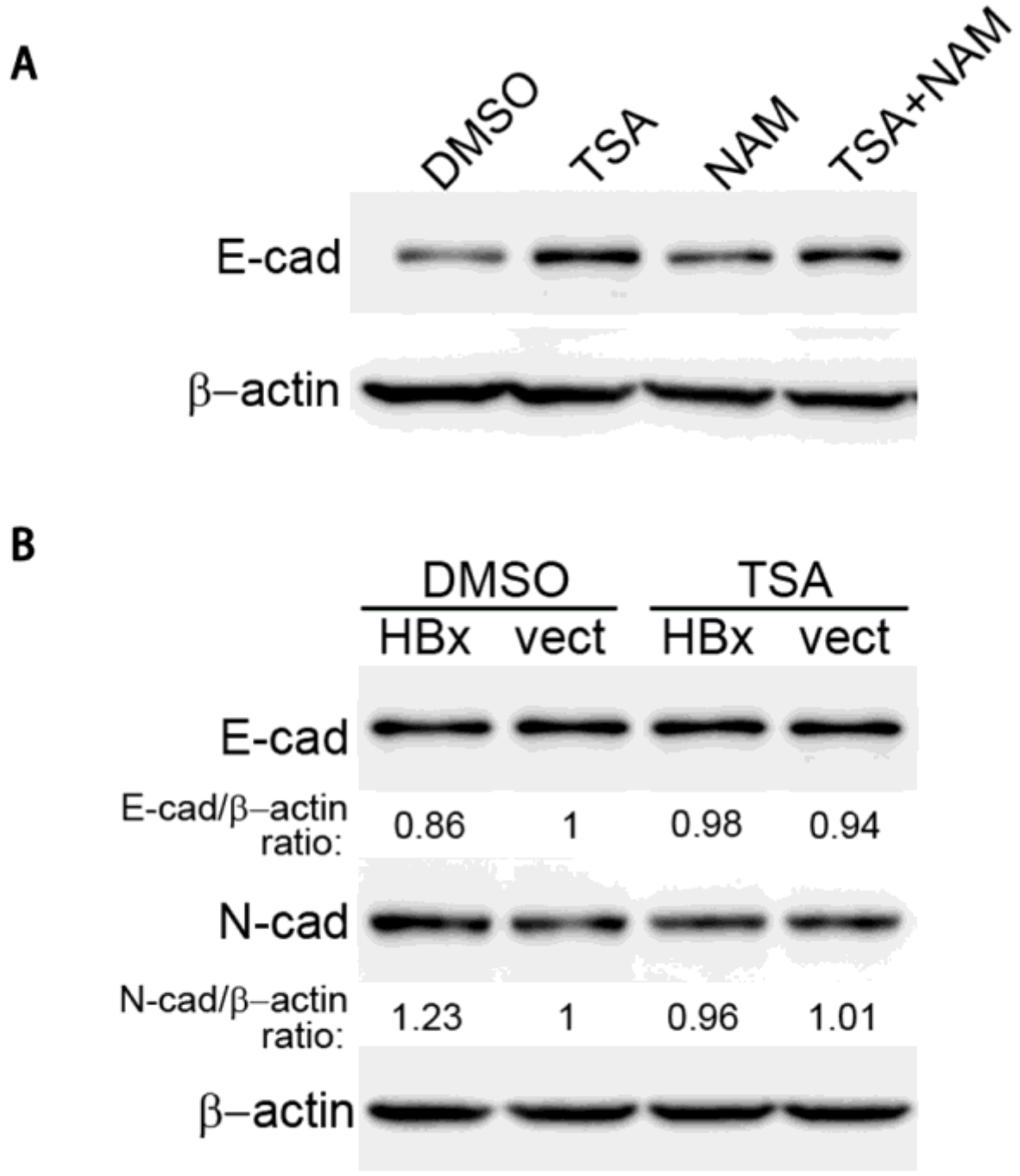

Figure 1

TSA inhibited HBx-enhanced metastasis in metabolic stress. (A) Immunoblot analysis of the expression of E-cad in Huh7 cells with TSA and/or NAM intervention for 16hr. Beta-actin were served as loading control. E-cad, E-cadherin. DMSO, solvent control. TSA, Trichostatin A. NAM, Nicotinamide. (B) Immunoblot analysis of the expression of E-cad and N-cad in Huh7 cells treated with EBSS starvation for 3hr. HBx and vect refered to the HBx-overexpressed group and the contorl group, respectively. Beta-actin were served as loading control. N-cad, N-cadherin. DMSO, solvent control group (16hr). TSA, TSA-treating group (16hr).

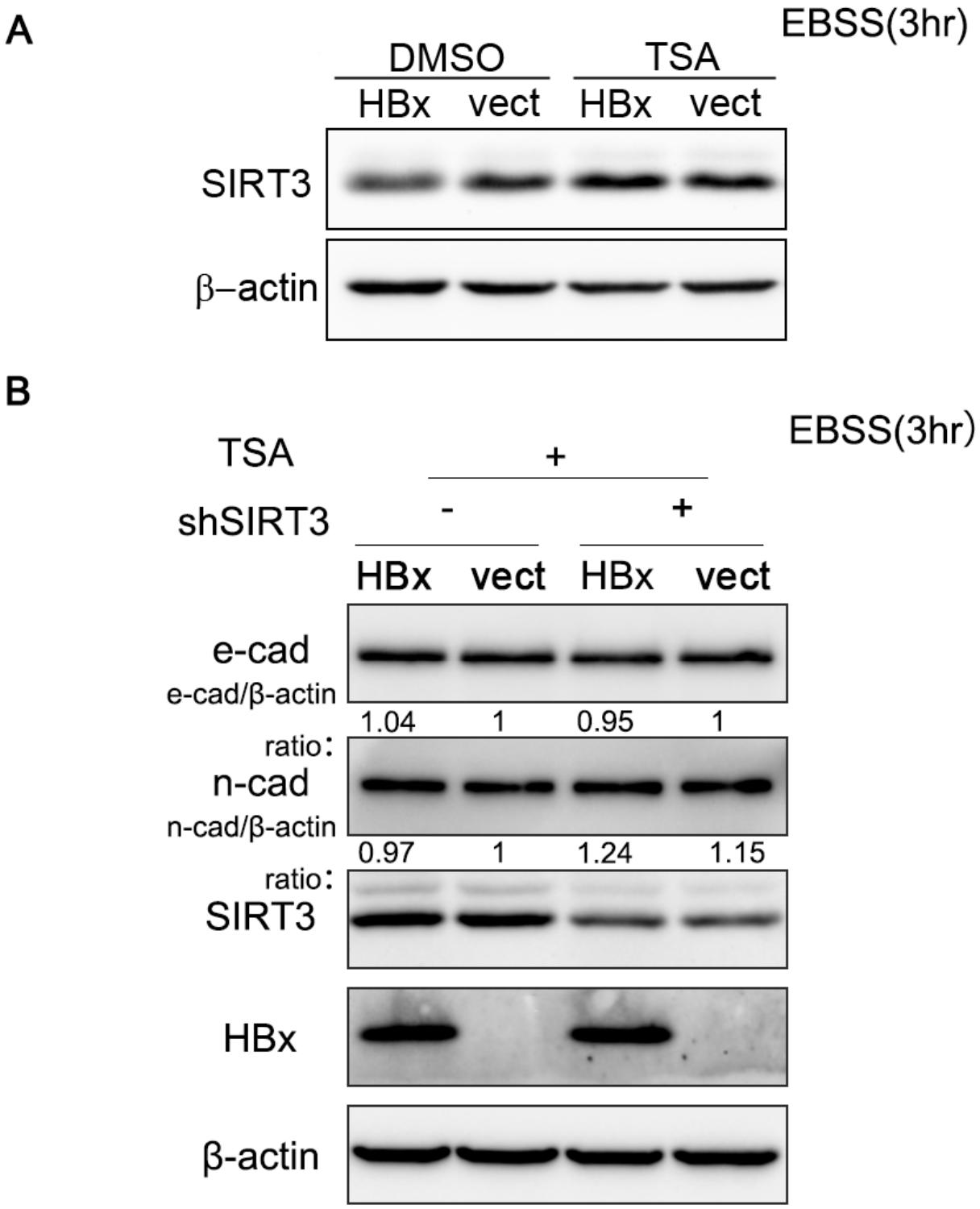

Figure 2

SIRT3 plays a role in TSA-related anti-cancer function in metabolic. (A) Immunoblot analysis of the expression of SIRT3 in Huh7 cells in metabolic stress. HBx and vect refer to the HBx-overexpressed group and the contorl group, respectively. Beta-actin were served as loading control. DMSO, solvent control group (16hr). TSA, TSA-treating group (16hr). (B) SIRT3 were knocked down in Huh7 cells mediated by

shSIRT3 lentivirus infection, or control NT-shRNA lentivirus. Immunoblot analysis of the expression of E-cad and N-cad in Huh7 cells in metabolic stress. Beta-actin were served as loading control.

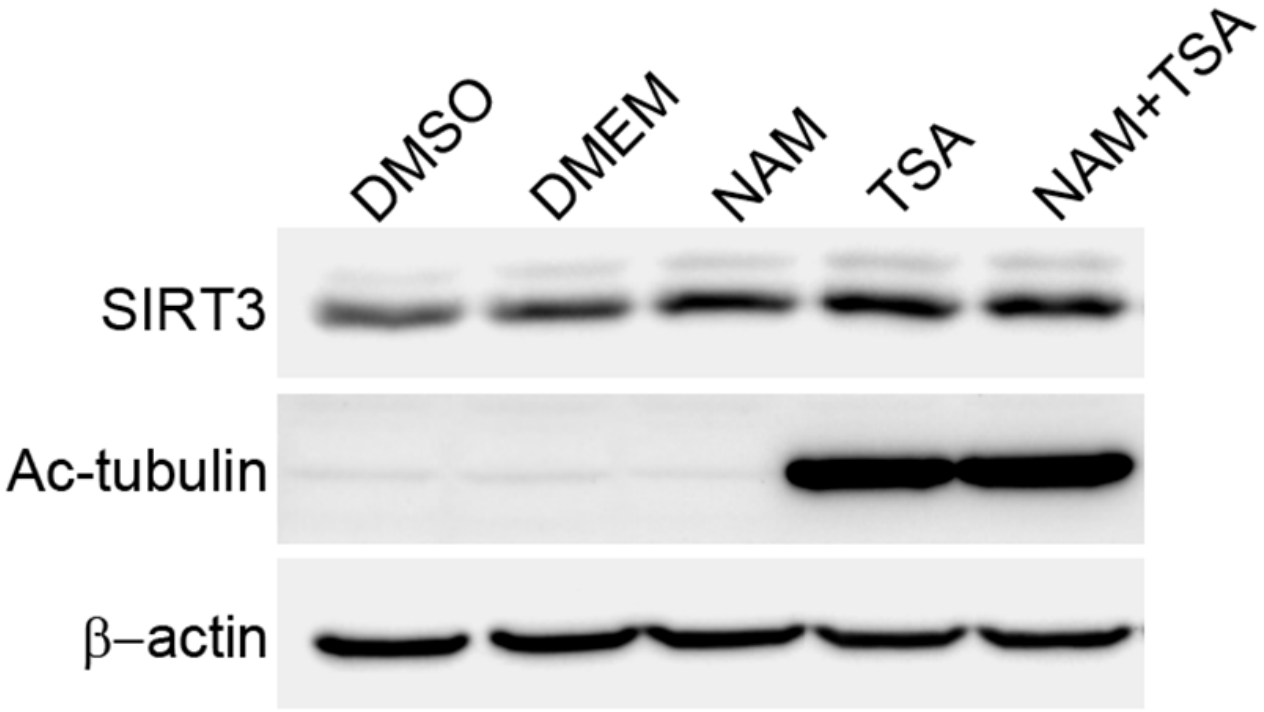

Figure 3

TSA promotes SIRT3 protein expression. Immunoblot analysis of the expression of SIRT3 in Huh7 cells with TSA and/or NAM intervention. Ac-tubulin and Beta-actin were served as positive control and loading control respectively. DMSO, solvent control group (16hr). DMEM, solvent control group. NAM, NAM-treating group (16hr). TSA, TSA-treating group (16hr).

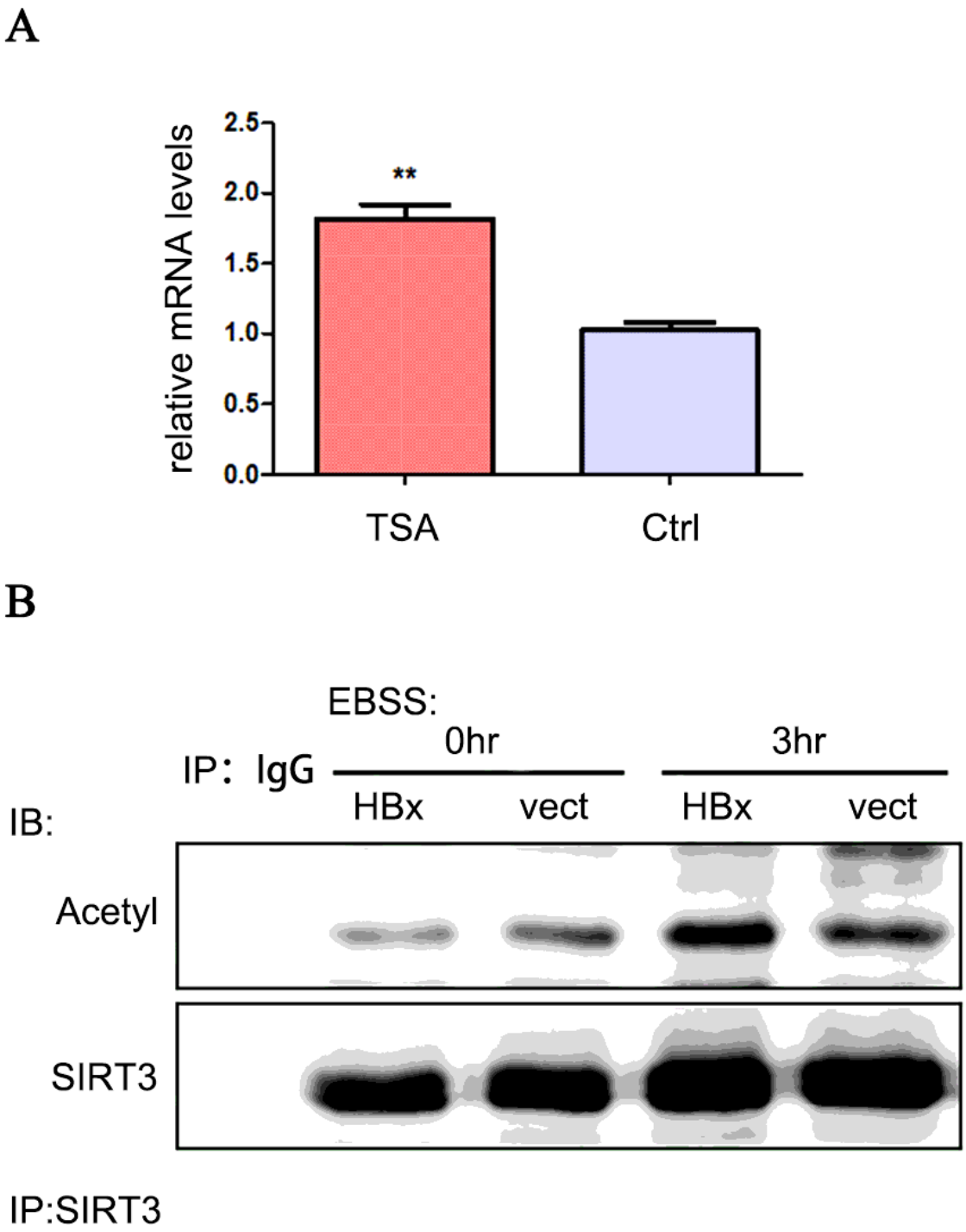

Figure 4

TSA induces SIRT3 transcriptional expression. (A) Quantification of mRNA levels of SIRT3 by real-time PCR in Huh7 cells in metabolic stress. TSA, TSA-treating group (16hr). Ctrl, solvent control group (16hr). (B) Immunoprecipitation analysis of the acetyl level of SIRT3 in Huh7 cells treated with or without EBSS starvation for 3hr. HBx and vect refer to the HBx-overexpressed group and the contorl group, respectively. IgG were served as negative control. IP, immunoprecipitation. IB, immunoblot.