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# Involvement of transcription factor XBP1s in the resistance of HDAC6 inhibitor Tubastatin A to superoxidation via acetylation-mediated proteasomal degradation

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ABSTRACT

HDAC6 is a major cytoplasmic deacetylase. XBP1s is a basic-region leucine zipper (bZIP) transcriptional factor. Despite their mutual involvement in the anti-oxidative process, there are no reports about their inter-protein interactions so far. Here we identified a direct link between HDAC6 inhibition and XBP1s transcription activity in anti-oxidative damage. We showed that the specific HDAC6 inhibitor Tubastatin A could up-regulate XBP1s transcriptional activity, thereby increasing anti-oxidative genes expression. Moreover, knock down of XBP1s could significantly abolish the cell growth protection afforded by Tubastatin A. We hypothesize that Tubastatin A acts to increase XBP1s protein levels that are dependent on its HDAC6 deacetylase inhibition via a mechanism involving acetylation-mediated proteasomal degradation, providing novel mechanistic insight into the anti-oxidative effects of HDAC6 inhibition.



## 1. Introduction

HDAC6 is the principal cytoplasmic deacetylase in mammalian cells [1]. HDAC6-specific substrates are varied, and include α-tubulin [2], cortactin [3], HSP90 [4], IFNαR [5], peroxiredoxin (Prx) I and Prx II [6]. Its involvement in deacetylation gives HDAC6 an important role in the progression of neurodegenerative diseases and lends itself to being a potential therapeutic target [6–8].

In addition to deacetylation, high levels of reactive oxygen damage cells are also believed to be associated with neurodegenerative disorders [9]. A potential interplay between deacetylation and oxidative stress can be found in data using Tubacin [6] and Tubastatin A [10], both highly selective HDAC6 inhibitors that have also showed good anti-oxidative activity.

Despite this prior work, the mechanism behind the anti-oxidative activity of HDAC6-specific inhibitors has still not been clarified. Two substrates have been found to be directly regulated by HDAC6: the cytoplasmic antioxidants enzymes peroxiredoxin (Prx) I and Prx II both appear to be involved in the anti-oxidative effects of HDAC6 inhibition [6]. Consistent with HDAC6 localization to the cytoplasm and its ability to deacetylate a range of cytoplasmic target proteins, it has been suggested that the effects of HDAC6 inhibition occur through a transcription-independent, local mechanism [6].

To this end, our work provide evidence that XBP1s, a bzip transcription factor that is involved in the mammalian unfolded protein response (UPR), could play an important role in the antioxidative activity of HDAC6 inhibition caused by Tubastatin A. This putative interaction between HDAC6 and nuclear transcription factor XBP1s provides evidence for a transcriptionally-regulated mechanism for HDAC6 function.

## 2. Materials and methods

### 2.1. Reagents and antibodies

Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen (Grand Island, New York, USA). Protein A-Agarose, anti-acetyl-tublin, and anti-β-actin antibody were purchased from Sigma–Aldrich (St. Louis, MO, USA). Anti-tubulin antibody was purchased from Epitomics (Burlingame, CA, USA) and Anti-acetyl-histone H3 antibody was from Millipore (Billerica, MA, USA). Anti-acetylated-lysine, anti-histone H3, anti-Flag and anti-HA antibodies were provided by Cell Signaling Technology (Danvers, MA, USA). Tubastatin A, SAHA and valproic acid (VPA) are gifts from Prof. Jingkang Shen (*Shanghai Institute of Materia Medica*). Tubacin was obtained from BioVision

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(Milpitas, CA, USA) and Niltubacin was from Enzo Life Sciences, Inc (Farmingdale, NY, USA).

### 2.2. Cell culture and cell proliferation assay

HEK293T and PC12 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, PR China). Cells were grown in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 95% air and 5% carbon dioxide ($CO_2$) at 37 °C. Cell growth was assessed using an MTT assay as described previously [11].

### 2.3. RNA extraction and Q-PCR

Total RNA was isolated using the TRIzol reagent (Invitrogen), according to the manufacturer's instructions. Reverse transcription and Q-PCR were conducted using the PrimeScript RT reagent and the SYBR Premix Ex TaqTM kits (Takara, China) at the ABI 7300 PCR system. Refer to Table 1 for relevant PCR primer sequences. All genes of interest were normalized to a GAPDH loading control and subsequent mRNA results were expressed as fold changes relative to GAPDH using the $2^{-\Delta\Delta CT}$ method [12].

### 2.4. Plasmids and transfection

The pFLAG-XBP1s-CMV2, pcDNA3-FLAG-HDAC6 and HA-Ubiquitin [13] were obtained from Addgene (Cambridge, MA, USA). XBP1s point mutants were generated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Santa Clara, CA, USA). Refer to Supplemental Table 1 for XBP1s point mutant primer sequences. The XBP1s siRNA sequence was synthesized by Shanghai GenePharma Co., Ltd. and was as 5′-gtctgctgagtccgcagca-3′ [14]. Ad-XBP1s was a gift from Dr. Yong Liu (The Institute for Nutritional Sciences (INS), Shanghai Institutes for Biological Sciences, CAS). Cell transfection was performed using lipofectamine™ 2000 (Invitrogen) according to the manufacturer's protocol.

### 2.5. Western Blot (WB)

Cells were lysed, separated using SDS–PAGE, and were immunoblotted with the related primary antibodies. Resulting bands were detected using HRP-conjugated secondary antibodies and ECL reagent (GE Healthcare Life Science, Pittsburgh, PA, USA) according to the manufacturer's instructions. The quantification of band intensities was performed using *SmartView* software in conjunction with the FR-980A Gel Image Analysis System (Shanghai Furi Science and Technology Co., Ltd., Shanghai, PR China).

**Table 1**
Q-PCR primer sequences for the interested genes.

| Gene | Forward primer (5′–3′) | Reverse primer (5′–3′) |
|---|---|---|
| *PRDX5* | GGCAGCATAGCCGGATCGGTG | CTCTGCCTGCCACGCACGTC |
| *CAT* | CCAGACACTCACCGCCACCG | TGGTAGTTGGCCACGCGAGC |
| *PDIA5* | AAGGCTGCCACCCAAGTGCG | GCAGATGGTGGGGTAGCCACG |
| *SOD1* | TTCGTTTCCTGCGGCGGCTT | GGACCGTCGCCCTTCAGCAC |
| *TRX-1* | GGCCGCTGCGGGAGACAAG | AGGTCCACACCACGTGGCAGA |
| *NQO-1* | GCCGCCTGAGCCCGGATATTG | AATGGCGGGCACCCCAAACC |
| *ERO1a* | TGGGGCTTGCTCGTCGGACT | CAGAAACACCGCTGTGCCGC |
| *EDEM1* | TGGCTTCATCGGCCAAGGCG | CCACCGCCGCCCAAAGCATA |
| *SEC61a* | TGGGTCCGCCACACCTCAGT | CCTGTGTCGGGGCAAGTGGC |
| *GAPDH* | GGGGCTCTCTGCTCCTCCCTG | CCAGGCGTCCGATACGGCCA |
| *XBP1s* | GAGTCCGCAGCAGGTGCAGG | GGGTCCAACTTGTCCAGAATGCCC |
| *XBP1u* | GAGTCCGCAGCACTCAGACTACG | GGGTCCAACTTGTCCAGAATGCCC |

### 2.6. Immunoprecipitation for the acetylated XBP1s detection and acetylation site identification

HEK-293T cells that were overexpressed Flag-XBP1s were lysed in RIPA buffer. Cell extracts were then immunoprecipitated with Protein A-Agarose beads conjugated to the anti-Flag antibody. The precipitated complexes were then analyzed through WB with either an anti-acetylated-Lysine or an anti-Flag antibody.

Acetylation site identification was performed by separating the previously precipitated IP sample through SDS–PAGE. A band of approximately~55 kDa was recovered and completely digested with trypsin. Spectrometric identification was performed using matrix-assisted laser desorption/ionization-time of flight mass spectrometry analysis (MALDI-TOF MS) and the final peptide mapping analysis was performed using the Mascot tool.

### 2.7. Co-immunoprecipitation to detect XBP1s ubiquitination state

For the co-immunoprecipitation of Flag-XBP1s and HA-Ubiquitin, HEK293T cells were co-transfected with Flag-XBP1s and HA-Ubiquitin plasmids. Cell lysates were then immunoprecipitated using Protein A-Agarose beads conjugated to the anti-Flag antibody. Precipitated complexes were then immunoblotted with both the anti-HA and anti-Flag antibodies.

### 2.8. Statistical analyses

Data are presented as means with standard deviation (±SD). Statistical analyses between groups were performed using two-tailed Student's *t* test with GraphPad software. $P < 0.05$ was considered as statistically significant.

## 3. Results

### 3.1. Tubastatin A up-regulates anti-oxidative gene expression related to transcription factor XBP1s

Hydrogen peroxide ($H_2O_2$) was used to induce oxidative stress and neuronal damage. Tubastatin A, an HDAC6 inhibitor known to have neuroprotective effects [10], was used to inhibit HDAC6 activity. Prior work has elucidated genes important to human anti-oxidant responses, which include peroxidases, superoxide dismutases and thiol redox regulating genes [15]. Based on this prior characterization, peroxidase genes *PRX5* and *CAT*, superoxide dismutase gene *SOD*, thiol redox regulating genes *TRX-1* and *PDIA5*, and Nrf2 target gene *NQO-1* were selected to investigate the hypothesis that HDAC6 inhibition affects the transcription level of anti-oxidative genes.

First, Tubastatin A was found to significantly reverse $H_2O_2$-induced inhibition of PC12 cell growth (Fig. 1A). This reversal was evident at both the 24 and 48 h marks, indicating that Tubastatin A could protect PC12 cells from $H_2O_2$-induced oxidative damage. Six anti-oxidative stress-related genes were detected with Q-PCR in cells treated with Tubastatin A and $H_2O_2$. Tubastatin A treatment did not affect the expression of *SOD*, *TRX1* and *NQO-1*, but significantly increased the expression of *CAT*, *PRDX5* and *PDIA5* genes. This increase was significant, particularly after $H_2O_2$ treatment (Fig. 1B). These findings suggest that HDAC6 inhibition could regulate some anti-oxidative genes expression.

All three up-regulated genes, *CAT*, *PRDX5* and *PDIA5*, have been reported to be regulated by transcription factor X-box binding protein-1 (XBP1s) [16]. This finding prompted us to speculate that the transcriptional activity of XBP1s had also increased. Previous research on XBP1s has shown that it regulates ERAD genes such as *EDEM1, SEC61α* and *ERO1α*. All three of these genes were

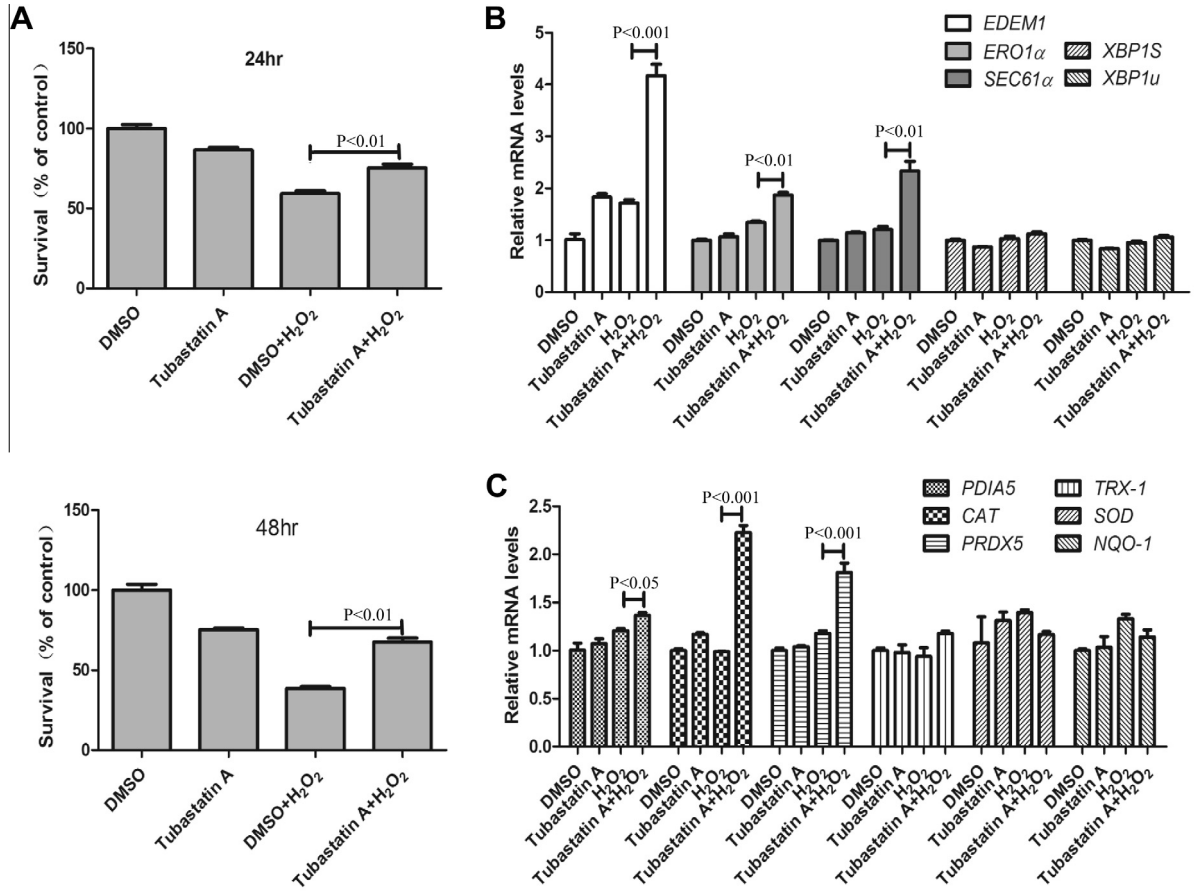


**Fig. 1.** Tubastatin A, HDAC6 selective inhibitor, regulated anti-oxidative genes transcription expression in $H_2O_2$ treated PC12 cells. (A) Tubastatin A protected PC12 cells growth from $H_2O_2$ treated for 24 or 48 h. (B) Q-PCR detection for anti-oxidative genes expression in PC12 cells after Tubastatin A treatment for 4 h. (C) Q-PCR detection for the XBP1s regulated ERAD related genes expression after Tubastatin A treatment for 4 h.

detected and, as Fig. 1C illustrates, the mRNA levels of all of them were increased with both Tubastatin A and $H_2O_2$ treatments. These results indicate that the transcription factor activity of XBP1s was up-regulated. However, we could not determine an effect on the transcription and splicing of *XBP1* since we found no visible changes in the transcription levels of either *XBP1u* or *XBP1s*.

### 3.2. XBP1s is involved in the transcriptional regulation of anti-oxidative genes and cell growth protection resulting from HDAC6 inhibition

The up-regulation of anti-oxidative stress and ERAD genes and their relationship to XBP1s (Fig. 1B and C) allowed us to hypothesize that XBP1s might be involved in the function of HDAC6 inhibition. Using siRNA for XBP1, we then knocked down XBP1 expression by approximately half (Fig. 2A). Concomitant to this knockdown, we observed that the previously reported up-regulation of *CAT*, *PRDX5* and *PDIA5* genes as a result of Tubastatin A and $H_2O_2$ treatment had dropped back to basal levels (Fig. 2B). This indicated that XBP1 was involved in the transcriptional regulation of anti-oxidative genes relating to HDAC6 inhibition. Additionally, we found that the down-regulation of *XBP1* resulted in a decrease in the protective effects of PC12 cells treated with both Tubastatin A and $H_2O_2$ when compared to controls (Fig. 2C). These results are the first to demonstrate the importance of XBP1s in the anti-oxidative activity of Tubastatin A.

### 3.3. Tubastatin A up-regulates XBP1s protein levels that are dependent on HDAC6 inhibition

Application of Tubastatin A had no effect on either the transcription or splicing of XBP1 (Fig. 1C). We then sought to determine if transcriptional activity up-regulation might be the result of increases in XBP1s protein expression. Since endogenous XBP1 has a rapid turnover due to proteasomal degradation [17,18], we used exogenous XBP1 to determine the relationship between Tubastatin A and XBP1.

Hek293T cells were transfected with Flag-tagged XBP1s, treated with Tubastatin A or the pan-HDAC inhibitor SAHA, and then examined with WB by using anti-Flag antibody. Treatment with both Tubastatin A and SAHA were found to increase XBP1s protein levels in a dose- and time-dependent manner (Fig. 3A and B).

Although Tubastatin A is the specific inhibitor of HDAC6, it was necessary to confirm the role of HDAC6 in the upregulation of XBP1s by Tubastatin A. Thus, Hek293T cells were co-transfected with the XBP1s-containing plasmid and Flag-tagged wild type HDAC6. Cells were lysed and the Flag-XBP1s levels were examined with WB after four hours of Tubastatin A treatment. In addition to the increase in wild type HDAC6 protein levels, the increase of exogenous XBP1s induced by Tubastatin A was reduced (Fig. 3C). These results indicate that HDAC6 is involved in Tubastatin A-induced XBP1s protein level increases. Consistent with this finding, over-expression of wild type HDAC6 also decreased exogenous XBP1s protein expression (Fig. 3D).

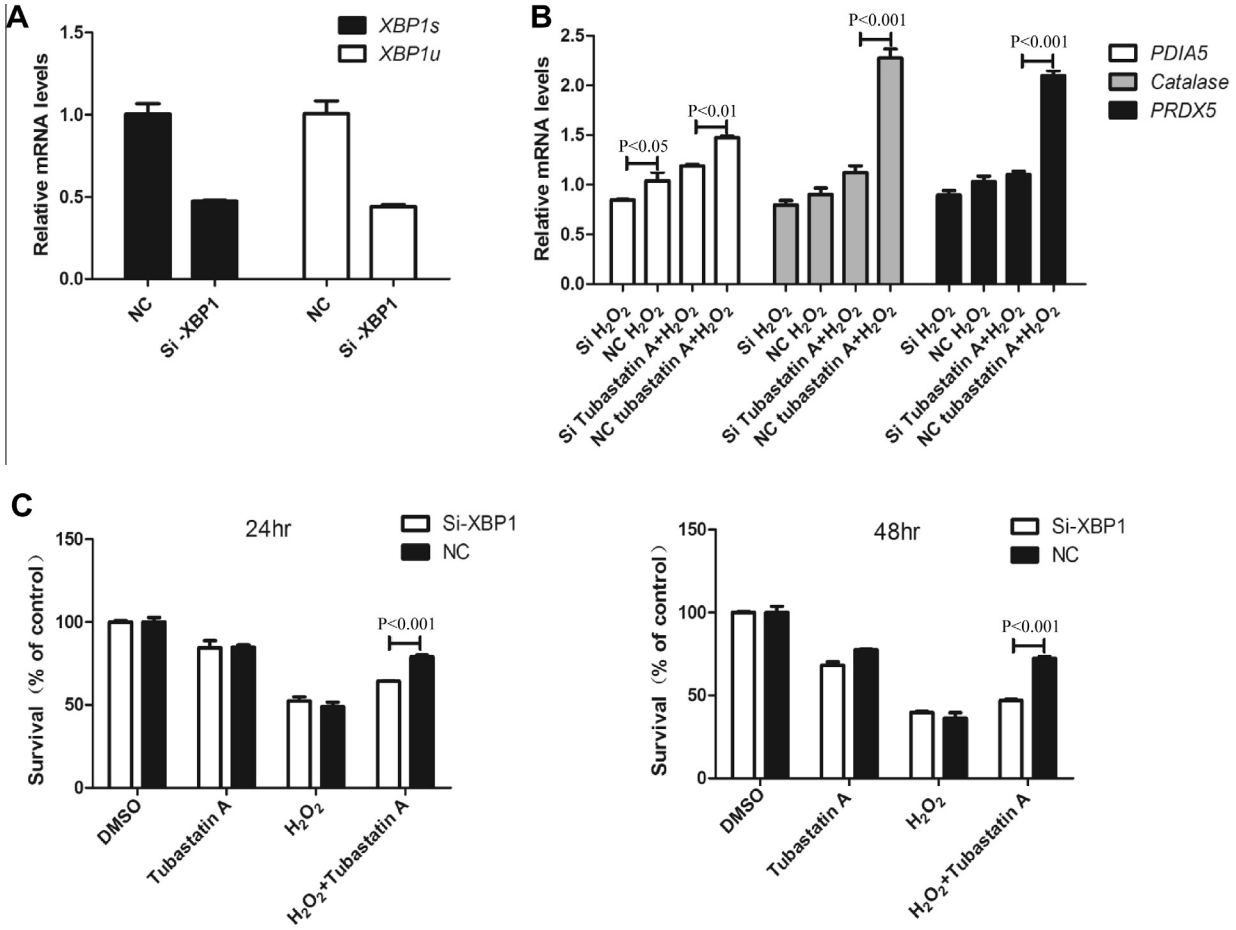


**Fig. 2.** XBP1s was involved in the transcriptonal regulation of antioxidative stress genes and cell growth protection of HDAC6 inhibition. (A) Q-PCR detection for XBP1 RNAi efficiency after transfected for 48 h. (B) Q-PCR detection for the *CAT*, *PRDX5* and *PDIA5* genes, when *XBP1s* was knocked down and treated with Tubastatin A for 4 h. (C) PC12 cell transfected with XBP1 siRNA and treated with $H_2O_2$ and Tubastatin A for 24 or 48 h were tested with MTT assay.

To test whether other HDAC6 inhibitors could have similar effect on XBP1s protein regulation, Tubacin, the selective HDAC6 inhibitor and Niltubacin, the inactive counterpart of Tubacin were used. Hek293T cells transfected with Flag-XBP1 plasmids were treated with Tubacin or Niltubacin for the indicated concentrations and time points. Tubastatin A was used as positive control. As shown in Fig. 3E, treatment with 2 or 4 μM Tubacin for 4 or 6 h could significantly enhance the tublin acetylation and the Flag-XBP1s protein levels, similar with Tubastatin A. As expected, Niltublin has no effect on both tublin acetylation and Flag-XBP1s protein level. This result indicated that regulation of XBP1s protein levels by Tubastatin A or Tubacin depended on inhibition of HDAC6.

### *3.4. Tubastatin A delays XBP1s protein degradation via acetylation-mediated proteasomal degradation*

Our data implicate the involvement of Tubastatin A in up-regulation of XBP1s. Slowing XBP1s degradation might be a possible mechanism behind Tubastatin A's actions. To determine whether Tubastatin A affects the half-life of XBP1s protein levels, Hek293T cells expressing Flag-XBP1s were treated with 100 μg/mL of a protein synthesis inhibitor, cycloheximide (CHX), to block further protein synthesis. The results showed that Tubastatin A treatment had slowed down the degradation kinetics compared to the control group (Fig. 4A). The half-life of XBP1s has been reported to be less than 10 min [21], which is similar to our findings. Furthermore, the half-life of XBP1s could be extended to approximately 30 min after Tubastatin A treatment (Fig. 4A and B). These results confirm that Tubastatin A is capable of postponing XBP1s degradation.

We then want to understand the role of ubiquitination with regards to Tubastatin A application. HA-Ubiquitin plasmid was co-transfected with Flag-XBP1s into Hek293T cells. After culturing for 24 h, cells were lysed with RIPA buffer, subjected to co-IP with anti-FLAG antibody, and then analyzed by WB using anti-FLAG and anti-HA antibodies. As shown in Fig. 4C and D, after treatment with either Tubastatin A or SAHA, the level of poly-Ub attachment to XBP1s was significantly decreased compared to controls. These data indicate that protein degradation was delayed during the attachment of poly-ubiquitin to the XBP1s protein.

There are many possible mechanisms for how lysine acetylation can inhibit proteasomal-mediated protein degradation [19]. However, competition between lysine acetylation and ubiquitination is the most simple and direct mechanism. Thus, the effect of Tubastatin A treatment on the XBP1s acetylation was evaluated. Hek293T cells were transfected with Flag-tagged XBP1s and treated with Tubastatin A or SAHA for 4 h. Cells were lysed with RIPA buffer, immunoprecipitated with anti-FLAG antibody, and then analyzed by WB with anti-FLAG and anti-acetylated-lysine antibodies. As indicated in Fig. 3A, XBP1s protein levels were increased in Tubastatin A or SAHA treated cells. Moreover, the acetylation status of XBP1s was significantly increased in Tubastatin A or SAHA treated samples (Fig. 4E). This indicates that XBP1s protein stability

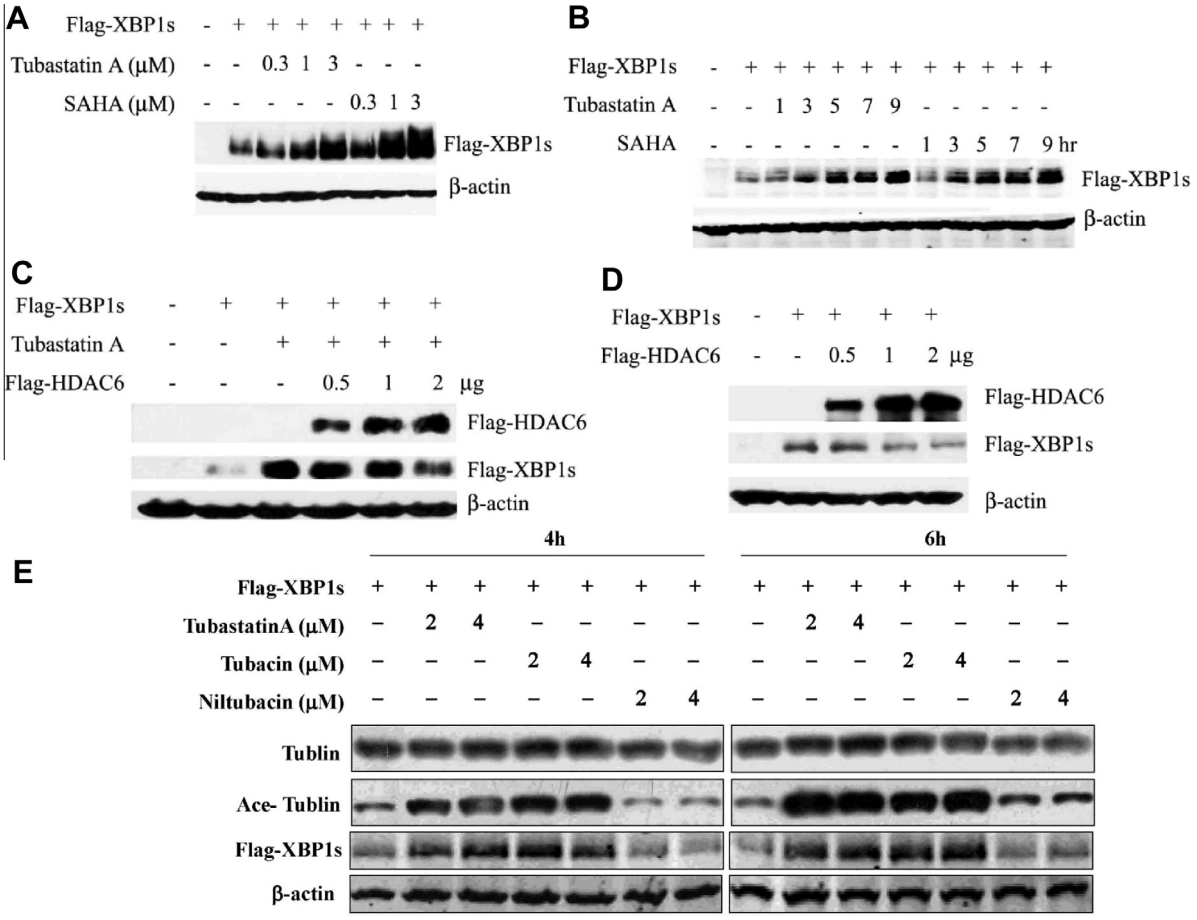


**Fig. 3.** Tubastatin A up-regulated XBP1s protein level dependent on HDAC6 inhibition. HEK293T cells were transfected with Flag-XBP1s plasmid and treated with SAHA or Tubastatin A at the indicated doses (A) and time points (B), and analyzed by WB with anti-Flag antibody. (C) Over-expression of HDAC6 partially reversed the up-regulation of Flag-XBP1s protein induced by Tubastatin A. HEK293T cells were co-transfected with Flag-XBP1s plasmid and increasing amounts of Flag-HDAC6, cultured for 24 h, and treated with Tubastatin A for 4 h, then detected by WB with anti-Flag antibody. (D) Wild type HDAC6 reduced the Flag-XBP1s protein level. HEK293T cells were co-transfected with Flag-XBP1s plasmid and increasing amounts of Flag-HDAC6, and detected by WB with anti-Flag antibody. (E) HEK293T cells transfected with Flag-XBP1s were treated with Tubastatin A, Tubacin, or Nitubacin at indicated doses for indicated time points, followed by WB for Ace-tublin and Flag-XBP1s.

regulated by Tubastatin A is relative to the balance between lysine acetylation and ubiquitination modifications.

## 4. Discussion

Our understanding of acetylation has long been limited, having been restricted to understanding the processes inside the nucleus (e.g. histones) [20] or to non-histone nuclear transcription factors [21]. It took the discovery of HDAC6 as a microtubule-associated deacetylase for researchers to realize that acetylation is not exclusively located to the nucleus [2]. Now, more and more cytoplasmic proteins have been found to be acetylated and involved in diverse cellular processes [22].

Since HDAC6 is a cytoplasmic deacetylase, many of its substrates include not only cytoplasmic proteins, but also membrane proteins such as EGFR [23] and GRK2 [24]. Furthermore, the mechanism of HDAC6 action seems to occur locally and not related with the nucleus or nuclear processes like transcription [6]. However, its mechanism is not entirely clear, as HDAC6 could enter nucleus and bind to Survivin proteins [25]. The Class I histone deacetylases HDAC 1, 2 and 3 are mainly localized to the nucleus, and could also localize to the endoplasmic reticulum and interact with GRP78 to modulate the unfolded protein response [26]. Possible mechanisms appear paradoxical and disordered, thus underscoring the complexity of acetylation regulation. Here, we pursue the possibility of HDAC6 interacting with nuclear transcriptional regulation through a chemical biology strategy. We found that HDAC6 could regulate anti-oxidative gene expression by regulating the protein stability of transcription factor XBP1s, which could resist oxidative stress in varying cell types and conditions [27,28]. This finding (Figs. 1 and 2) extends the HDAC6 sphere of influence from a local cytoplasmic environment to nuclear transcription. This potentially implicates other transcriptional factors or nuclear proteins involved in the functioning of HDAC6.

HDACs are classified into four classes depending on sequence identity and domain organization. These include Zn-dependent histone deacetylases (class I, II, IV) and the class III NAD-dependent deacetylase SIRT family [29]. XBP1s could be directly deacetylated by SIRT1, one of class III NAD-dependent deacetylase SIRT family, and SIRT1 inhibitor EX-527 could up-regulate XBP1s protein levels [30]. To this end, VPA, the multiple HDACs from class I and class II (but not HDAC6 or HDAC10) inhibitor [31] was used to evaluate the specificity of HDAC6 on XBP1s protein. Hek293T cells transfected with Flag-XBP1s were treated with different HDAC inhibitors (SAHA, VPA and Tubastatin A) for 4 h and analyzed with WB for the H3 and α-tublin acetylation and XBP1s protein (Fig. S1). SAHA was found to increase both histone H3 and tubulin acetylation protein levels, Thus, VPA could only increase the acetylation of

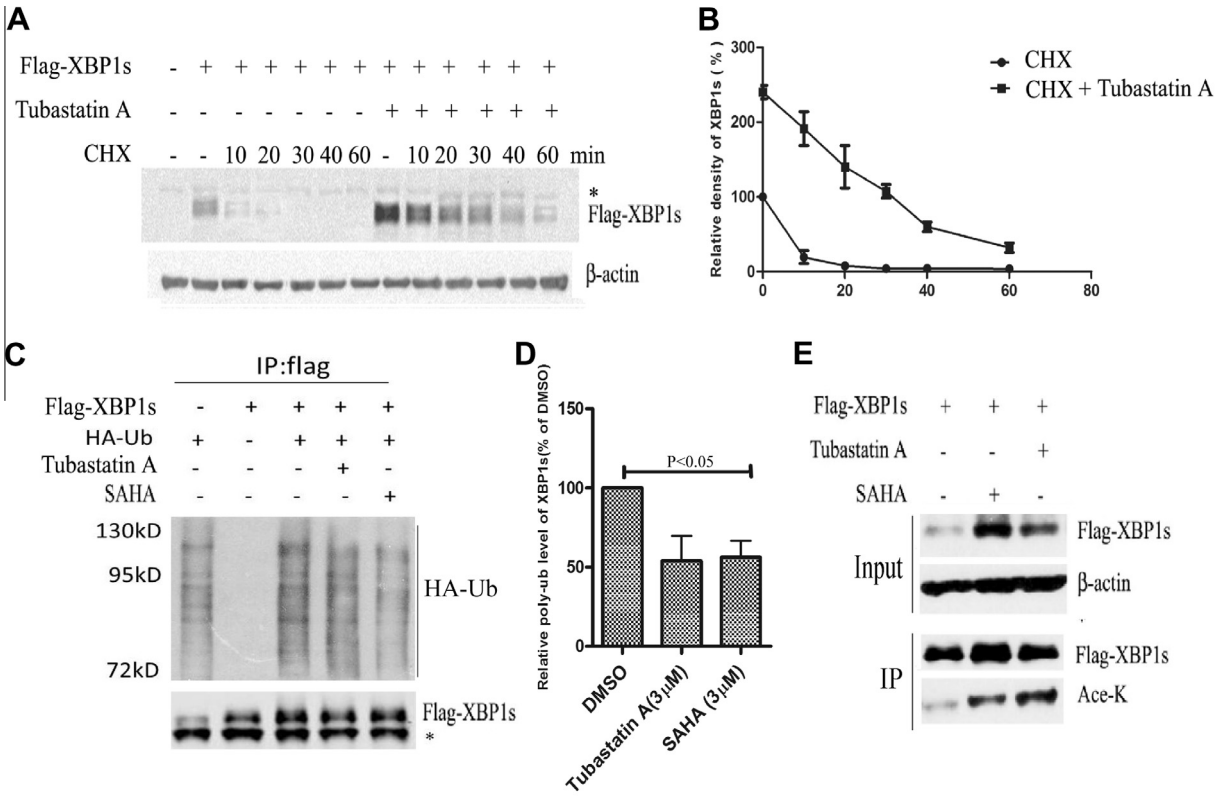


**Fig. 4.** Tubastatin A delayed XBP1s protein degradation via acetylation-mediated proteasomal degradation. (A) HEK293T cells transfected with Flag-XBP1s plasmid, cultured for 24 h, and treated with 3 μM Tubastatin A for 4 h before treatment with cycloheximide (100 μg/ml). Cells were lysed at the indicated time points and immunoblotted with anti-Flag antibody. (B) The relative intensity of Flag-XBP1s proteins at the indicated time points was quantified using *SmartView* software and standardized to 100% for the cycloheximide-pretreated (CHX 0 h) sample without Tubastatin A treatment in three independent experiments. (C) HEK293T cells were co-transfected with Flag-XBP1s and HA-Ub plasmids, cultured for 24 h, and treated with 3 μM Tubastatin A or SAHA for 4 h. Cell lysates were subjected to anti-Flag antibody immunoprecipitation (IP) and analyzed by WB with anti-Flag, HA and β-actin antibodies. (D) The relative intensities of HA-Ub levels were quantified using *SmartView* and standardized to 100% for the DMSO control in 2 independent experiments. (E) SAHA or Tubastatin A treatment increased the acetylation of Flag-XBP1s. HEK-293 cells transfected with Flag-XBP1s were treated with SAHA or Tubastatin A for 4 h. Cell lysates were subjected to immunoprecipitation (IP) and analyzed by WB with anti-acetylated lysine, Flag and β-actin antibodies.

H3, while Tubastatin A only increased tubulin acetylation. Both SAHA and Tubastatin A were found to increase XBP1s expression in a dose-dependent manner, but VPA treatment had no obvious effect on XBP1s protein levels. These results indicate that class I and class II members of the HDAC family have no effect on XBP1s protein expression.

As Fig. 4 shows, Tubastatin A can increase XBP1s protein stability which was linked to the shift of lysine ubiquitination to lysine acetylation. A direct competition between lysine acetylation and ubiquitination has been proposed as a major regulatory mechanism preventing protein ubiquitination and degradation, such as p53 and HIF1α [32,33]. To better understand this competition, we sought to find the acetylation sites of XBP1s. Hek293T cells were transfected with Flag-XBP1s, treated with SAHA for 4 h, and then immunoprecipated with an anti-Flag antibody. The acetylation sites of Flag-XBP1s were then analyzed via mass spectrometry. Numerous sites were identified (data not shown). Among them, lys241, 257, 276 and 297 sites were identified, all of which have decent evolutionary conservation (Fig. S2A and B). To validate their necessity, all four sites were then mutated into arginines. To detect whether Tubastatin A could affects the half-life of XBP1s mutant protein levels, Flag-XBP1s mutant was transfected into Hek293T cells and treated with CHX and/or Tubastatin A, as previously described. Cells were lysed and analyzed with WB. However, Tubastatin A still could delay mutant XBP1s degradation and increase mutant XBP1s protein levels (Fig. S2C). We conclude that these four sites are not responsible for XBP1s protein stability and that the actual sites responsible for XBP1s stability require further examination. However, since the complexity of acetylation-dependent mechanisms that control protein stability is becoming even more evident, it is possible that this process might proceed through unexpected mechanisms not involving lysines or directly affecting the activity of ubiquitination machinery and of the proteasome itself [19].

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.05.134.

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