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ORIGINAL ARTICLE

PTEN activation through K163 acetylation by inhibiting HDAC6 contributes to tumour inhibition

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Phosphatase and tensin homologue deleted on chromosome 10 (PTEN), an important tumour-suppressor gene, is mutated, downregulated or dysfunctional in many tumours. The phosphatase activity of PTEN depends on membrane translocation (activation). As promising anti-cancer agents, histone deacetylase (HDAC) inhibitors, particularly trichostatin A (TSA), can promote PTEN membrane translocation, but the underlying mechanism remains unknown. In this study, we revealed that non-selective HDAC inhibitors, namely, TSA or suberoylanilide hydroxamic acid (SAHA), induced PTEN membrane translocation through PTEN acetylation at K163 by inhibiting HDAC6. K163 acetylation inhibited the interaction of the PTEN C-tail with the remaining part of PTEN, resulting in PTEN membrane translocation. Overexpression of wild-type PTEN, but not K163-mutated PTEN, facilitated the inhibition of cell proliferation, migration and invasion, as well as xenograft tumour growth, induced by SAHA or tubastatin A, an HDAC6-specific inhibitor. These results indicated that PTEN activation by inhibiting HDAC6 significantly contributed to tumour inhibition. Therefore, non-selective HDAC or HDAC6-specific inhibitors may be more clinically suitable to treat tumours without PTEN mutations or deletions.

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INTRODUCTION

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is an important tumour-suppressor gene.[1–3] Deletions and mutations in PTEN were observed in many cancers, including brain, breast and prostate cancers,[1–2] as well as in primary endometrial carcinoma,[4] melanoma,[5] and head and neck cancer.[6] PTEN dephosphorylates phosphatidylinositol-3,4,5-trisphosphate (PIP3) to negatively regulate the PI3K/AKT pathway. Functional inactivation of PTEN upregulates PI3K/AKT signals and enhances protein synthesis, cell migration and tumour-induced angiogenesis.[7–11] PTEN is mainly found in the cytosol and nucleus, and a small fraction of PTEN is dynamically associated with the inner surface of the plasma membrane. Membrane translocation is critical for the tumour-suppressor function of PTEN, whose main substrate, PIP3, is located in the plasma membrane;[12] as such, this membrane-bound PTEN is the activated form.[12–15] PTEN contains a phosphatase domain in the N-terminal, a C2 domain and a C-terminal tail with 50 amino acids.[16–18] Each domain exhibits a certain function in relation to the membrane translocation of PTEN.[17] Binding of thioredoxin-1 to PTEN through a disulphide bond between the active site Cys32 of thioredoxin-1 and Cys212 of the C2 domain of PTEN inhibits PTEN membrane translocation and activation.[19] PTEN sumoylation at K266 promotes its membrane translocation.[20] A mutant PTEN with a deletion of the C-terminal tail (PTEN-ΔC) more strongly binds to the membrane, but this binding is weakened with co-expression of the C-terminal tail by directly interacting with PTEN-ΔC.[21] Triple mutations of E161A, K163A and K164A prevent PTEN-ΔC from interacting with the C-terminal tail and consequently enhance PTEN membrane translocation.[21]

Acetylation, a posttranslational modification of histones and non-histone proteins at lysine residues, has an important role in multiple processes, such as gene expression and protein activity.[22] PTEN can be acetylated at K125 and K128 by the p300/CBP-associated factor (PCAF), thereby decreasing PTEN activity.[23] Protein acetylation is regulated by histone acetyltransferases and histone deacetylases (HDACs), which can reverse lysine acetylation.[24]

Eighteen HDACs have been identified in humans, and these enzymes are divided into four classes: Class I (HDAC1, 2, 3 and 8), Class II (HDAC4, 5, 6, 7, 9 and 10), Class III (SIRT1, 2, 3, 4, 5, 6 and7) and Class IV (HDAC11 only).[22] Classes I, II and IV are collectively named as 'classical' HDACs. Knockout of specific HDACs shows that most Class I and II HDACs are necessary for mice to develop or survive without severe defects.[25] Meanwhile, mice lacking HDAC6 develop normally[26] and exhibit low AKT phosphorylation.[27] Inhibition of HDAC6 can enhance acetylation of several non-histone proteins,[28–30] inhibit oncogenesis[27] and reduce cell motility.[31,32] Inhibitors of HDACs, particularly classical HDACs, can induce the reexpression of silenced genes, cell growth arrest and apoptosis in various cancer cell lines.[33–38] As such, HDAC inhibitors function as promising anti-cancer agents. Suberoylanilide hydroxamic acid (SAHA) is the first HDAC inhibitor approved by the U.S. Food and Drug Administration for treatment of cutaneous T-cell lymphoma.[39] Different HDAC inhibitors target distinctively various HDAC targets. SAHA and trichostatin A (TSA) are non-selective inhibitors that target HDAC1–11; sodium butyrate (NaBu) partially targets HDAC1–9, except HDAC6; and tubastatin A and tubacin selectively target HDAC6.[22] Although several studies have shown that HDAC inhibitors arrest cell cycle and induce apoptosis mainly by targeting cell cycle-related factors, such as cyclin-dependent kinase inhibitor 1A (p21Waf1/Cip1)[40] and B-cell lymphoma/leukemia-2 (Bcl-2),[38] the mechanism underlying the anti-tumour effects of these inhibitors remains to be elucidated. We previously

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reported that TSA not only induces PTEN expression but also PTEN membrane translocation (activation).[41] However, the role and underlying mechanism of PTEN in the anti-tumour effect of HDAC inhibitors remain to be explored. Further investigations must focus on the following aspects: the relationship between PTEN membrane translocation induced by HDAC inhibitors and PTEN acetylation, specific HDACs that promote PTEN acetylation, the mechanism through which PTEN acetylation affects membrane translocation and the role of PTEN acetylation in the anti-tumour effect of HDAC inhibitors.

In this study, we revealed that HDAC inhibitors induced PTEN membrane translocation through PTEN acetylation at K163 by inhibiting HDAC6. PTEN activation by HDAC6 inhibition significantly contributed to tumour inhibition.

RESULTS

TSA or SAHA more efficiently induced PTEN membrane translocation than PTEN expression

We previously showed that TSA induced PTEN expression and membrane translocation in TCa83 Cells.[41] In the current study, we performed western blot analysis and determined that TSA or SAHA more efficiently induced PTEN membrane translocation than PTEN expression in 293T or SACC-83 cells (Figures 1a–c). This finding indicated that TSA or SAHA not only functioned as an inducer but also as an important activator of PTEN. TSA- or SAHA-induced membrane translocation of PTEN was directly observed under a confocal fluorescence microscope (Figure 1d).

TSA or SAHA induced PTEN acetylation at K163

Given that HDAC inhibitors can increase acetylation and regulate the functions of non-histone proteins,[22,42] we determined whether TSA or SAHA can increase PTEN membrane translocation (activation) through acetylation. The results of immunoprecipitation assay showed that acetylation of precipitated PTEN was significantly enhanced in 293T cells after treatment with TSA for 24 h compared with that in the control group, despite that the amount of precipitated PTEN in both groups were equal (Figure 2a). These results suggested that TSA could induce PTEN acetylation.

We then constructed PTEN deletion mutants and restricted the TSA-induced acetylation site of PTEN in the phosphatase domain (Figures 2b and c). Fourteen lysine residues (K6, K13, K60, K62, K66, K80, K102, K125, K128, K144, K147, K163, K164 and K183) were observed in the phosphatase domain of PTEN. We mutated each of the lysine residues including K125, K128, K6, K13, K60/62, K80 and K102 to arginine, but none of these mutations could eradicate TSA-induced PTEN acetylation (data not shown). Meanwhile, the mutation of K163 to arginine completely abolished TSA- and SAHA-induced PTEN acetylation (Figures 2d and e). Moreover, this mutation did not affect the baseline of PTEN acetylation compared with wild-type PTEN (Figures 2d and e). These results indicated that TSA or SAHA induced PTEN acetylation at K163.

TSA or SAHA induced PTEN membrane translocation through PTEN acetylation at K163

As shown in Figure 3a, the levels of membrane-bound endogenous PTEN and total endogenous PTEN in 293T cells transfected with EGFP-PTEN or EGFP-PTEN-K163R, as well as membrane-bound exogenous wild-type PTEN (EGFP-PTEN), increased after treatment with TSA compared with those in the corresponding vehicle groups. Moreover, the level of membrane-bound exogenous mutant PTEN (EGFP-PTEN-K163R) was not affected by TSA compared with that in the corresponding vehicle group. The expression levels of total exogenous PTEN were also comparable. Similar results were observed in PTEN-null U-87 MG cells transfected with wild-type EGFP-PTEN or EGFP-PTEN-K163R after treatment with TSA or SAHA for 24 h (Figures 3b and c). In 293T cells, TSA promoted membrane translocation of EGFP-PTEN, but not that of EGFP-PTEN-K163R, as observed under a confocal fluorescence microscope (Figure 3d). Furthermore, we mutated K163 to glutamine to mimic K163 acetylation and detected that EGFP-PTEN-K163Q more effectively translocated to the membrane than the wild type (Figures 3e and f).

TSA-induced K163 acetylation of PTEN inhibited the interaction of PTEN-ΔC with the C-terminal tail, resulting in enhanced PTEN membrane translocation

To explore the mechanism underlying PTEN membrane translocation induced through acetylation at K163, we co-expressed PTEN-ΔC or PTEN-K163R-ΔC with the C-terminal tail in 293T cells, which were then treated with TSA. As shown in Figure 4a, TSA significantly inhibited the interaction between PTEN-ΔC and the C-terminal tail, but not between PTEN-K163R-ΔC and the C-terminal tail. The interaction between PTEN-K163R-ΔC and the C-terminal tail was similar to that of PTEN-ΔC and the C-terminal tail in the absence of TSA. This finding suggested that the mutation of K163 into arginine did not affect the interaction between PTEN-ΔC and the C-terminal tail. Moreover, membrane translocation of both PTEN-ΔC and PTEN-K163R-ΔC were significantly inhibited in the presence of the C-terminal tail; TSA partially reversed the decrease in membrane translocation of PTEN-ΔC, but not that of PTEN-K163R-ΔC in the presence of the C-terminal tail (Figures 4b and d). We also mutated K163 of PTEN-ΔC into glutamine to mimic acetylation at K163 and observed that PTEN-K163Q-ΔC showed a lower affinity for the C-terminal tail than PTEN-ΔC (Figure 4e). In addition, membrane translocation of PTEN-K163Q was not affected by co-expression of Flag-C tail (Figure 4f).

Inhibition of HDAC6 enhanced PTEN acetylation and membrane translocation

To determine HDACs that are specifically involved in TSA- or SAHA-induced acetylation of PTEN, we used SAHA (non-selective inhibitor), NaBu (inhibitor for HDAC 1–9, except HDAC6), tubastatin A (HDAC6-specific inhibitor), or short interfering RNA (siRNA) for HDAC10 or HDAC11 to treat 293T cells. In contrast to NaBu and knockdown of HDAC10 or HDAC11, SAHA and tubastatin A enhanced acetylation of PTEN (Figures 5a–c). This finding suggested that only HDAC6 inhibition could enhance PTEN acetylation.

Acetylation and membrane translocation of PTEN were reduced by overexpression of HDAC6 but enhanced by knockdown of HDAC6 (Figures 5d and e). Interaction between PTEN and HDAC6 was also observed (Figure 5f).

Inhibition of HDAC6 enhanced PTEN acetylation at K163 and membrane translocation

To examine the relationship of PTEN acetylation and membrane translocation mediated by HDAC6 through acetylation at K163, we transfected EGFP-PTEN or EGFP-PTEN-K163R into PTEN-null U-87 MG or PTEN-null NCI-H1650 cells. The cells were then treated with a HDAC6-specific inhibitor, namely, tubastatin A or tubacin, for 24 h. As shown in Figures 6a–d, acetylation and membrane translocation of EGFP-PTEN, but not EGFP-PTEN-K163R, were enhanced by treatment with tubastatin A or tubacin compared with those in the vehicle groups. By contrast, the amounts of the precipitated EGFP-PTEN and total EGFP-PTEN were comparable. Membrane translocation of EGFP-PTEN, but not EGFP-PTEN-K163R, induced by tubastatin A could be directly observed under a confocal fluorescence microscope (Figure 6e). Similar to tubastatin A and tubacin, knockdown of HDAC6 enhanced acetylation and

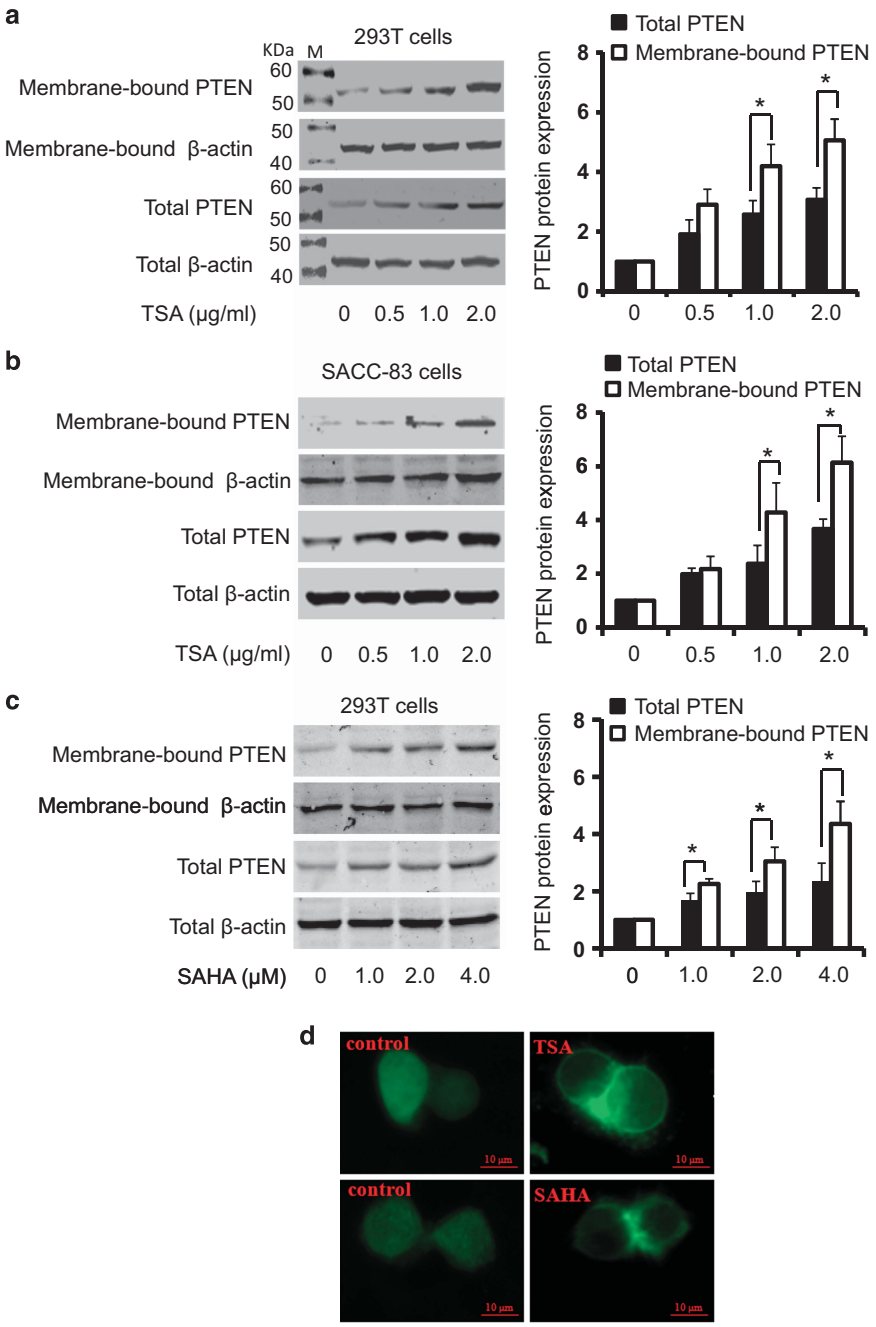


Figure 1. TSA or SAHA more efficiently induced PTEN membrane translocation than PTEN expression. (**a, b**) 293T or SACC-83 cells were treated with different doses of TSA for 24 h. Total protein and membrane protein were extracted and subjected to western blot analysis. The membranes were stripped for β-actin detection, which served as an internal control for equal loading. The target bands were scanned, and densitometry was performed. Data are presented as fold changes compared with the control group. (**c**) 293T cells were treated with different doses of SAHA for 24 h. The experiment was performed similar to that in **a** and **b**. (**d**) Fluorescent microphotographs of 293Tcells transfected with EGFP-PTEN and treated with TSA or SAHA for 24 h. *$P < 0.01$. M, marker.

membrane translocation of PTEN, but not EGFP-PTEN-K163R (Figures 6f and g).

PTEN, but not K163R mutant, facilitated SAHA-induced inhibition of U-87 MG cell migration and invasion

To determine the involvement of PTEN acetylation at K163 in cell migration and invasion inhibited by HDAC inhibitors, we performed transwell migration and invasion assays in U-87 MG cells stably transfected with wild-type PTEN or K163R mutant. As shown in Figures 7a–d, both SAHA and tubastatin A more significantly inhibited the migration and invasion of U-87 MG cells stably transfected with wild-type PTEN than that of U-87 MG cells stably transfected with PTEN-K163R or an empty vector.

PTEN, but not K163R mutant, facilitated SAHA- or tubastatin A-induced cell growth arrest and reduced AKT phosphorylation in U-87 MG cells

As shown in Figure 7e, the growth of U-87 MG cells stably transfected with PTEN did not differ from that of U-87 MG cells stably transfected with PTEN-K163R; nevertheless, both of their

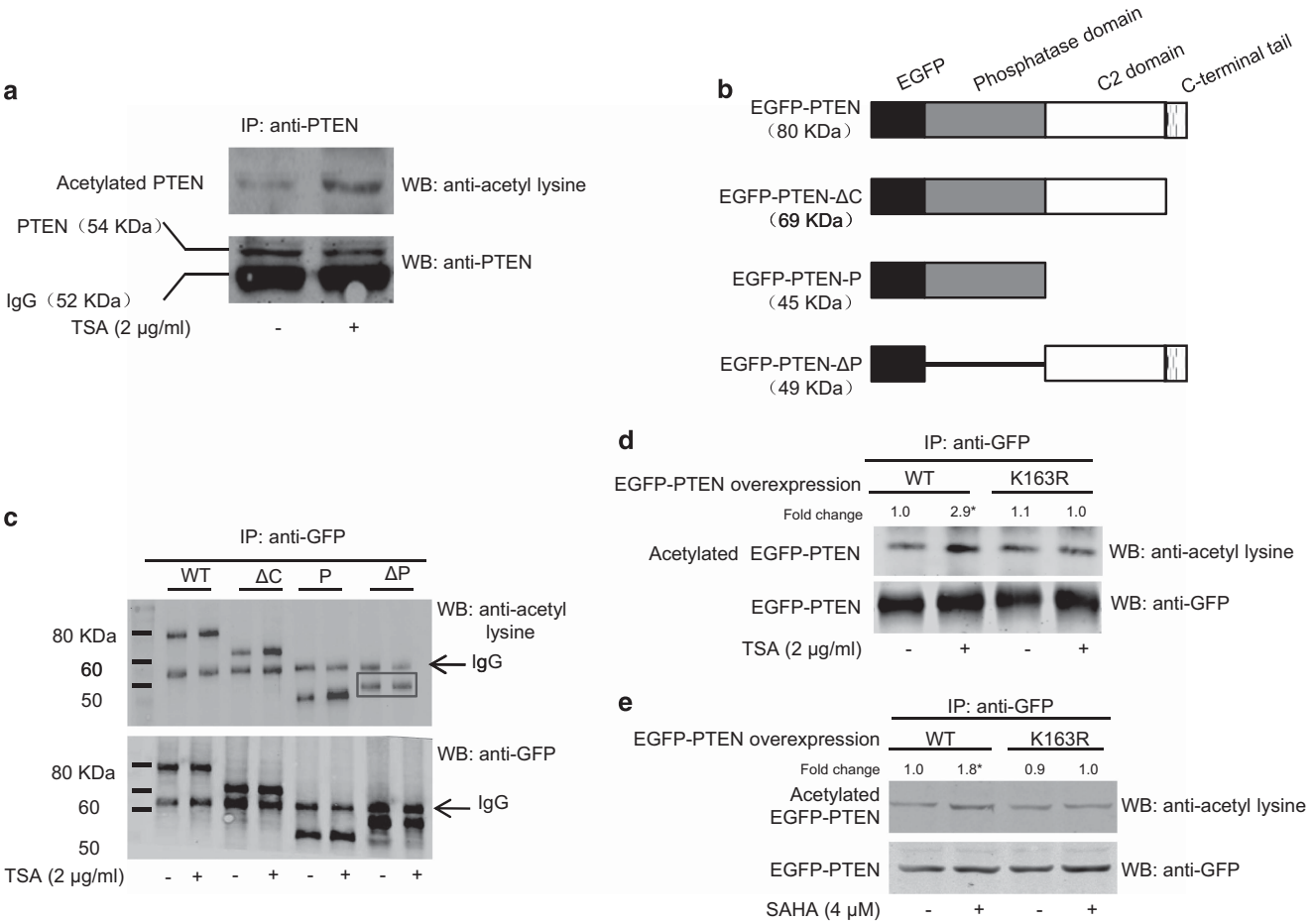


Figure 2. TSA or SAHA induced PTEN acetylation at K163. (**a**) TSA induced PTEN acetylation. 293T cells were treated with TSA for 24 h. Whole-cell lysates were immunoprecipitated and detected with anti-acetyl lysine antibody. (**b**) Schematic diagram of deletion mutants of PTEN. (**c**) Deletion of phosphatase domain abolished TSA-induced acetylation of the mutant of PTEN (indicated by the red rectangle). 293T cells were transfected with deletion mutants of PTEN and treated with TSA for 24 h. Whole-cell lysates were immunoprecipitated and subjected to detection with anti-acetyl lysine antibody. (**d, e**) Mutation of PTEN K163 into arginine abolished TSA- and SAHA-induced PTEN acetylation. 293T cells were transfected with wild-type EGFP-PTEN or mutant EGFP-PTEN-K163R for 24 h and then treated with TSA or SAHA for 24 h. Whole-cell lysates were immunoprecipitated and subjected to detection with anti-acetyl lysine antibody. The membrane was stripped for PTEN binding with anti-GFP antibody. $^*P < 0.01$. IP, immunoprecipitation; WB, western blot.

growth rates were slightly slower than that of U-87 MG cells stably transfected with an empty vector. SAHA more significantly inhibited the growth of U-87 MG cells stably transfected with PTEN (65% of non-treated U-87 MG cells stably transfected with PTEN, $P < 0.05$) than that of U-87 MG cells stably transfected with an empty vector (76% of non-treated U-87 MG cells stably transfected with an empty vector) and U-87 MG cells stably transfected with PTEN-K163R (82% of non-treated U-87 MG cells stably transfected with PTEN-K163R, $P < 0.05$). The phosphorylation (activation) of AKT coincided with the proliferation of cells in different treatments (Figure 7f). Similar results were observed in cells treated with tubastatin A (Figures 7g and h).

PTEN, but not K163R mutant, facilitated SAHA- or tubastatin A-induced inhibition of xenograft tumour growth

As shown in Figures 7i and j, the xenograft tumour weights of U-87 MG cells stably transfected with PTEN were considerably lower than that of U-87 MG cells stably transfected with PTEN-K163R or an empty vector after inoculation into nude mice and subsequent treatment with SAHA or tubastatin A for 3 weeks.

DISCUSSION

In this study, we presented evidence that non-selective HDAC inhibitors, namely, TSA and SAHA, could promote acetylation and membrane translocation of PTEN through PTEN acetylation at K163 and HDAC6 inhibition. The activated PTEN acetylation and membrane translocation inhibited cell proliferation, migration and invasion, as well as tumour growth.

PTEN acetylation at K163 was proposed as the mechanism underlying TSA- or SAHA-induced PTEN membrane translocation or activation. Non-selective HDAC inhibitors, namely, TSA and SAHA, more efficiently induced PTEN membrane translocation than PTEN expression in the cell lines tested. As membrane-bound PTEN is the activated form of PTEN,[43] these non-selective HDAC inhibitors were regarded as important activators of PTEN. Moreover, TSA- or SAHA-induced PTEN membrane translocation was dependent on K163 acetylation in the phosphatase domain of PTEN. To our knowledge, the present study is the first to demonstrate that acetylation at K163 could be a positive posttranslational modification of PTEN activity. The effect of acetylation at K163 on PTEN activity was opposite to that at K125 and K128, which are negative regulatory sites for PTEN activity.[23] More importantly, mutations of K125 and K128 in PTEN did not

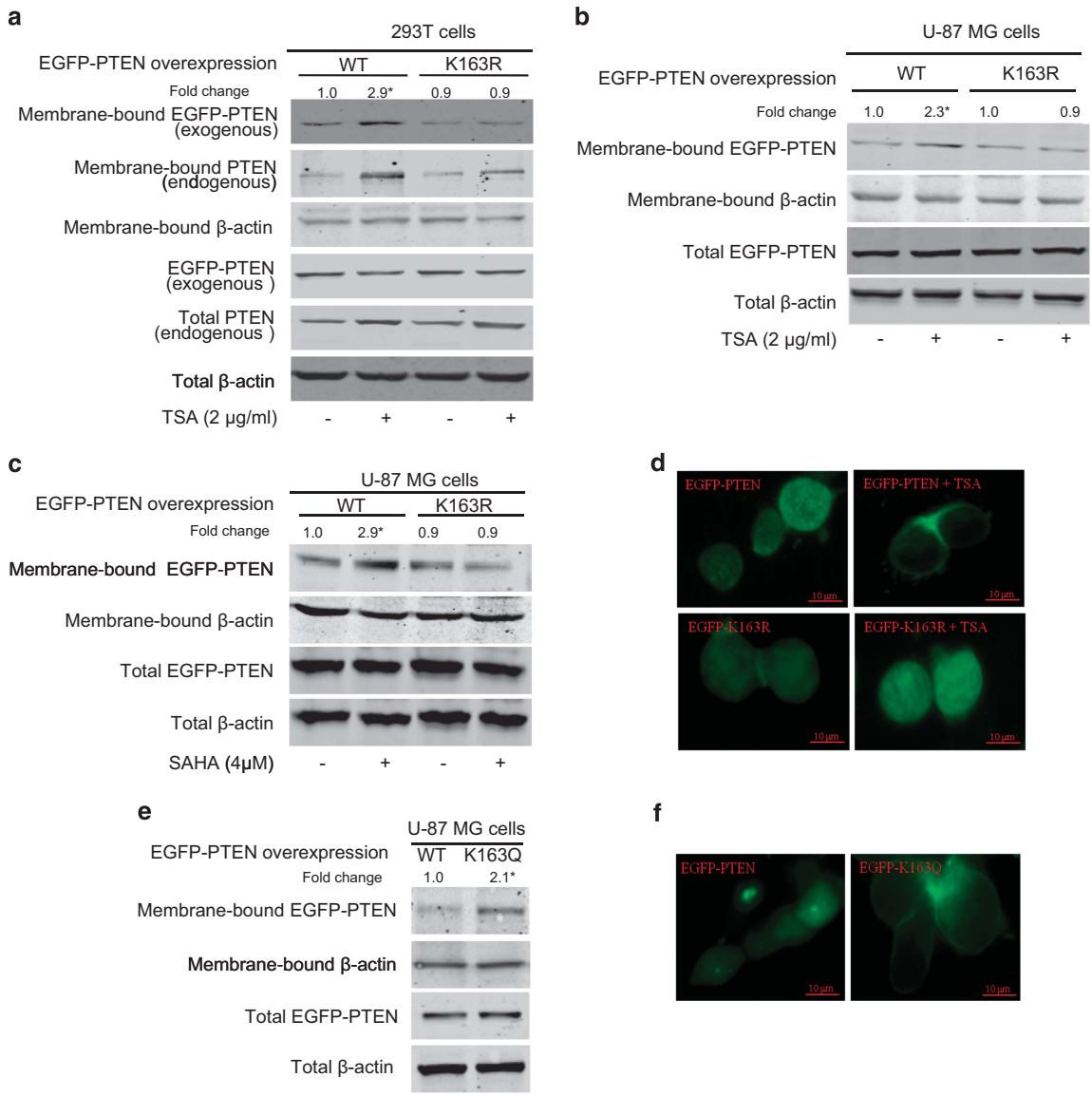


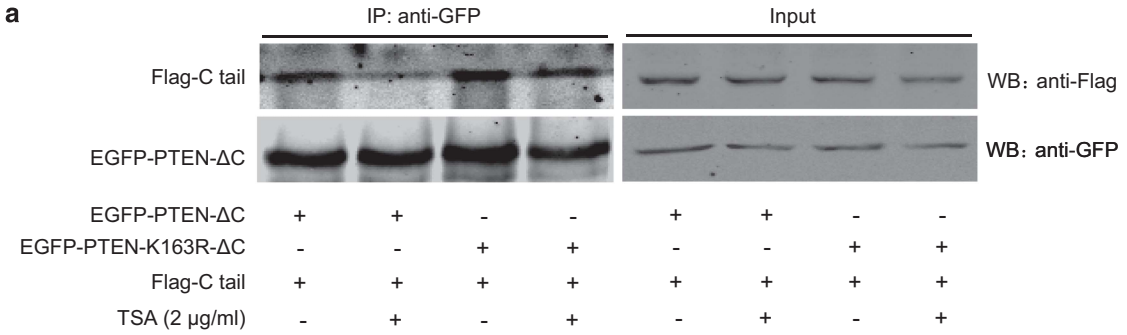
Figure 3. TSA or SAHA induced PTEN membrane translocation through PTEN acetylation at K163. (**a**) 293T cells were transfected with wild-type EGFP-PTEN or mutant EGFP-PTEN-K163R for 24 h and then treated with TSA for 24 h. Total protein and membrane protein were extracted, subjected to western blot and detected with antibodies as indicated (exogenous PTEN was detected by anti-GFP, whereas endogenous PTEN was detected by anti-PTEN antibody). (**b**, **c**) PTEN-null U-87 MG cells were transfected with wild-type EGFP-PTEN or mutant EGFP-PTEN-K163R for 24 h and then treated with TSA or SAHA for 24 h. Experiments were performed similar to that in **a**. (**d**) Fluorescent microphotographs of 293T cells transfected with EGFP-PTEN or EGFP-PTEN-K163R and treated with TSA for 24 h. Wild-type EGFP-PTEN, but not EGFP-PTEN-K163R, was translocated to the membrane after treatment. (**e**) Mutation of K163 of PTEN into glutamine enhanced PTEN membrane translocation. U-87MG cells were transfected with wild-type EGFP-PTEN or mutant EGFP-PTEN-K163Q for 48 h. Total protein and membrane protein were extracted, subjected to western blot and detected with anti-GFP antibody. (**f**) Fluorescent microphotographs of 293T cells transfected with wild-type EGFP-PTEN or mutant EGFP-PTEN-K163Q for 48 h. *$P < 0.01$.

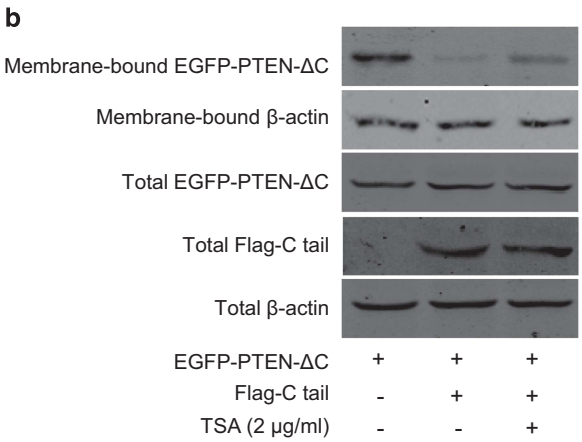
affect TSA- or SAHA-induced PTEN membrane translocation; hence, acetylation at these sites may exhibit minimal counteracting effect on TSA- or SAHA-induced PTEN activation. Of 14 lysine residues in the phosphatase domain of PTEN, mutation of 8 lysines did not affect the induction of PTEN acetylation by TSA. By contrast, K163 mutation could completely abolish the induction of PTEN acetylation and membrane translocation by TSA or SAHA. Considering the effect of K163 acetylation, the remaining five lysine residues were not mutated and tested, and thus excluded from studies on possible involvement in the induction of PTEN acetylation by TSA or SAHA. The degrees of acetylation or membrane translocation did not differ between wild-type PTEN and PTEN-K163R mutant, which indicated that the baseline of K163 acetylation contributed sparingly to the baseline of PTEN membrane translocation or activation or was tightly controlled. However, TSA or SAHA significantly enhanced K163 acetylation, resulting in PTEN activation. Therefore, K163 was considered an important positive regulatory site for PTEN activation and may function as a target for future development of anticancer drugs.

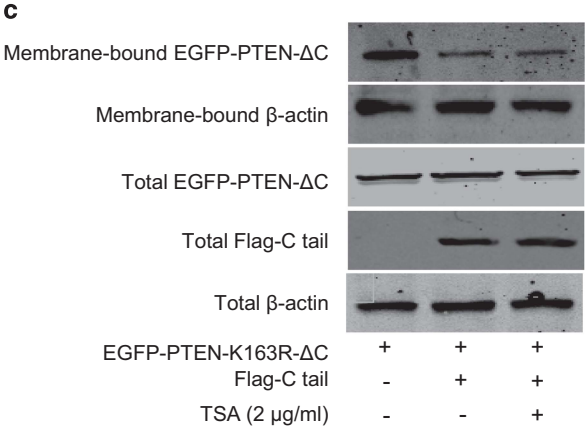
The mechanism underlying the induction of PTEN membrane translocation was determined as the inhibited interaction of PTEN-ΔC with its C-terminal tail by acetylation at K163. This interaction was previously demonstrated to negatively regulate PTEN membrane translocation.[21] Although the triple mutations of E161A, K163A and K164A also inhibit the interaction between PTEN-ΔC and its C-terminal tail,[21] we observed that the mutation of K163 to arginine alone did not affect the interaction. We revealed that such mutation abolished TSA-induced inhibited

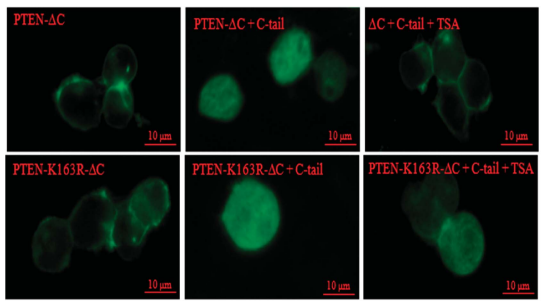
interaction between PTEN-ΔC and its C-terminal tail and, consequently, TSA-induced membrane translocation of PTEN-ΔC. These results confirmed that K163 acetylation reduced the interaction between PTEN-ΔC and the C-terminal tail, resulting in membrane translocation or activation of PTEN. Similarly, acetylation of p53 blocks the interaction of p53 with its repressor mouse double minute protein 2 (Mdm2), leading to p53 activation.[44] Acetylation of a specific lysine in a protein sequence can interfere with the interaction between proteins or peptides possibly because of changes in resultant charges, that is, from a positively charged lysine to a neutral one.

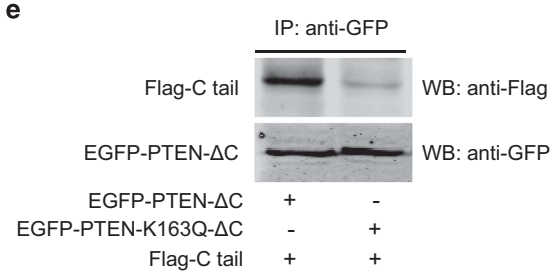
In this study, HDAC6 was demonstrated to be an important regulator of PTEN, particularly PTEN activation through K163 acetylation. In contrast to NaBu, non-selective HDAC inhibitors,

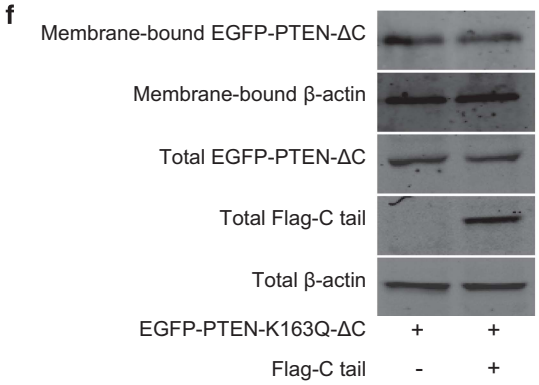












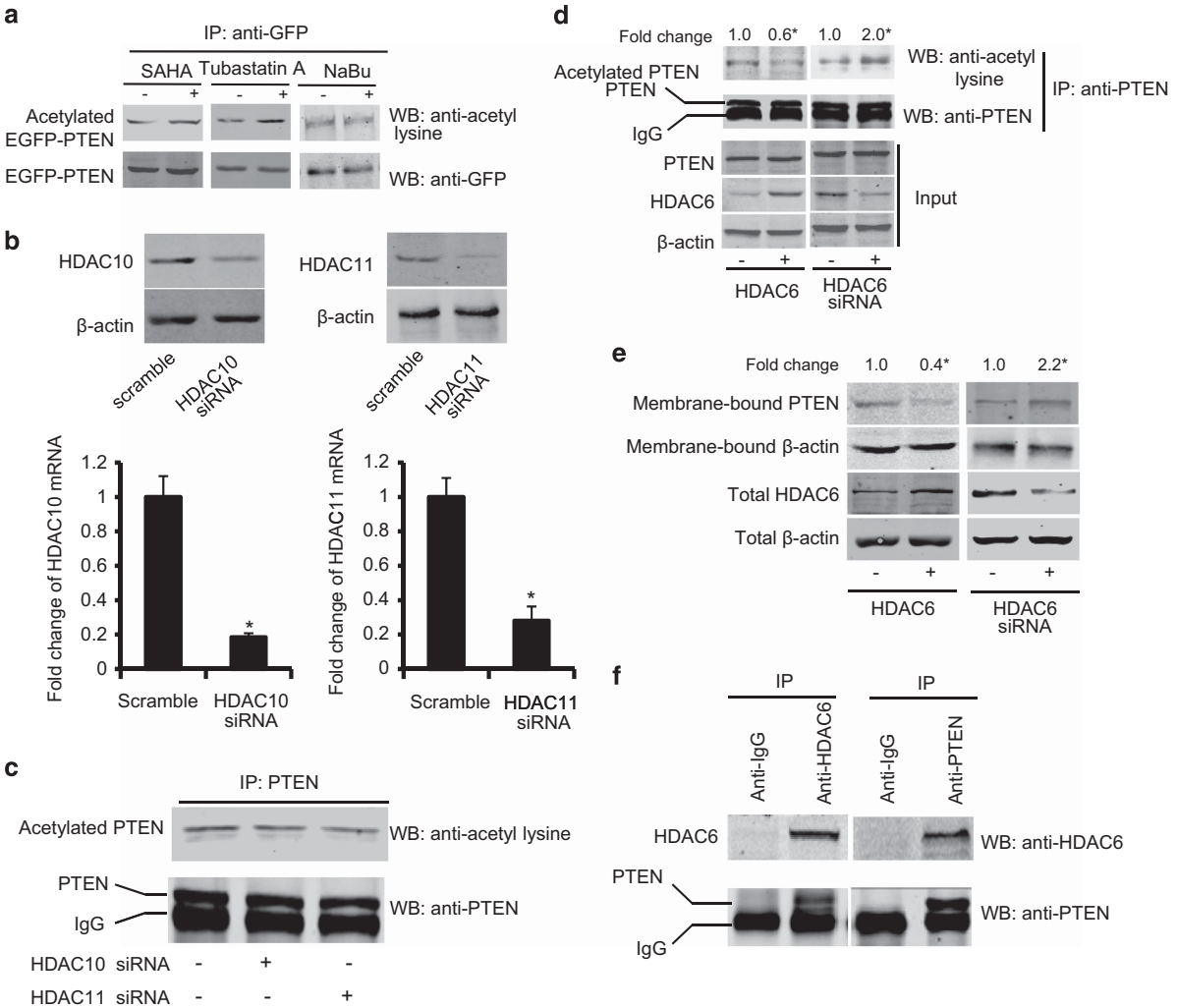


Figure 5. Inhibition of HDAC6 induced PTEN acetylation and membrane translocation. (**a**) Non-selective HDAC inhibitor SAHA (inhibitor for HDAC1–11) and HDAC6-specific inhibitor tubastatin A, but not NaBu (inhibitor for HDAC1–9, except HDAC6), induced PTEN acetylation. PTEN-null U-87 MG cells were transfected with EGFP-PTEN for 24 h and then treated with different inhibitors for 24 h. (**b**) Confirmation of knockdown of HDAC10 and HDAC11. 293Tcells were transfected with scrambled siRNA, HDAC10 or HDAC11 siRNA for 48 h. Protein expression was detected by western blot analysis, and mRNA expression was quantified through real-time PCR. (**c**) Knockdown of HDAC10 or HDAC11 did not affect PTEN acetylation. Specific siRNA for HDAC10 or HDAC11 was transfected into 293T cells for 48 h. Whole-cell lysates were immunoprecipitated with PTEN antibody and subjected to western blot with anti-acetyl lysine antibody. (**d**) Overexpression and knockdown of HDAC6 decreased and increased PTEN acetylation, respectively. 293T cells were transfected with HDAC6 or HDAC6 siRNA for 48 h; the cells and whole-cell lysates were immunoprecipitated with PTEN antibody and detected with acetyl lysine antibody. (**e**) Overexpression and knockdown of HDAC6 decreased and increased PTEN membrane translocation, respectively. 293T cells were transfected with HDAC6 or HDAC6 siRNA for 48 h. Total protein and membrane protein were subjected to western blot and detected with PTEN antibody. (**f**) HDAC6 interacted with PTEN. Cell lysates of 293T cells were subjected to co-immunoprecipitation assay with either anti-IgG, anti-PTEN or anti-HDAC6 and subsequently allowed to bind to anti-PTEN or anti-HDAC6. *$P < 0.01$. IP, immunoprecipitation; WB, western blot.

Figure 4. Acetylation of K163 inhibited the interaction between PTEN-ΔC and its C-terminal tail, resulting in PTEN membrane translocation. (**a**) TSA treatment resulted in the inhibition of interaction of PTEN-ΔC, but not of PTEN-K163R-ΔC, with its C-terminal tail. 293T cells were co-transfected with Flag-C-terminal tail and EGFP-PTEN-ΔC or EGFP-PTEN-K163R-ΔC and treated with TSA for 24 h. Cell lysates were immunoprecipitated with anti-GFP antibodies, subjected to western blot and detected with anti-Flag or anti-GFP antibodies. (**b, c**) TSA treatment partially blocked the C-terminal tail-induced inhibition of the membrane translocation of PTEN-ΔC, but not that of PTEN-K163R-ΔC. 293T cells were co-transfected with Flag-C terminal tail and EGFP-PTEN-ΔC or EGFP-PTEN-K163R-ΔC and treated with TSA for 24 h. (**d**) Fluorescent microphotographs of 293T cells co-transfected with Flag-PTEN C-terminal tail and EGFP-PTEN-ΔC or EGFP-PTEN-K163R-ΔC and treated with TSA for 24 h. Both PTEN-ΔC and PTEN-K163R-ΔC were mainly located in the membrane in the absence of the C-terminal tail and diffused in the whole-cell in the presence of the C-terminal tail. TSA treatment promoted membrane translocation of PTEN-ΔC, but not that of PTEN-K163R-ΔC, in the presence of the C-terminal tail. (**e**) Mutation of K163 into glutamine to mimic acetylation of K163 (PTEN-K163Q-ΔC) showed a lower affinity for C-terminal tail than PTEN-ΔC. 293T cells were co-transfected with Flag-C-terminal tail and EGFP-PTEN-ΔC or EGFP-PTEN-K163Q-ΔC. Cell lysates were immunoprecipitated with anti-GFP antibodies and detected with anti-Flag or anti-GFP antibodies. (**f**) PTEN C terminal tail did not reduce membrane translocation of PTEN-K163Q-ΔC. 293T cells were co-transfected with Flag-C terminal tail and EGFP-PTEN-ΔC or EGFP-PTEN-K163Q-ΔC for 48 h. IP, immunoprecipitation; WB, western blot.

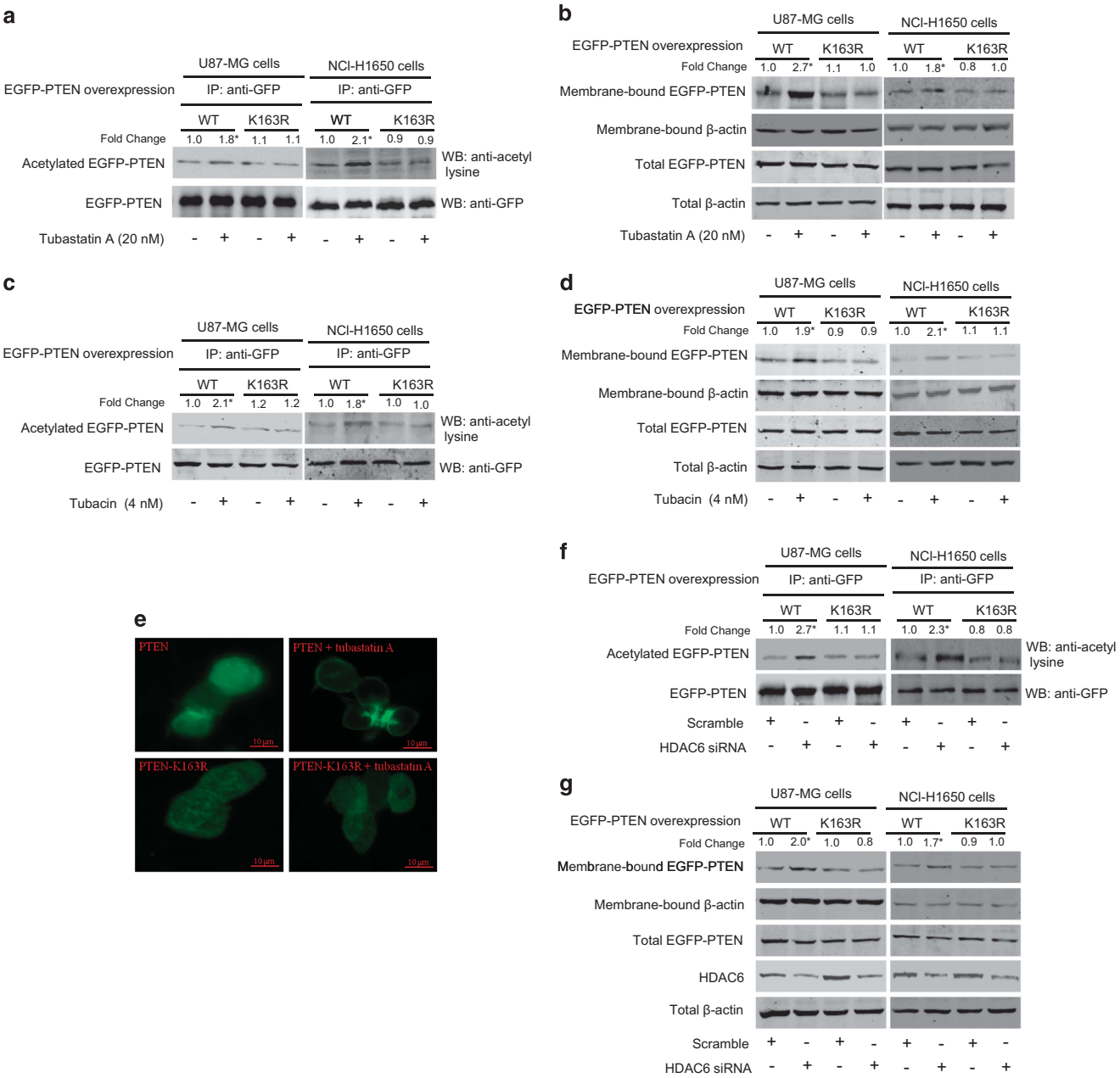
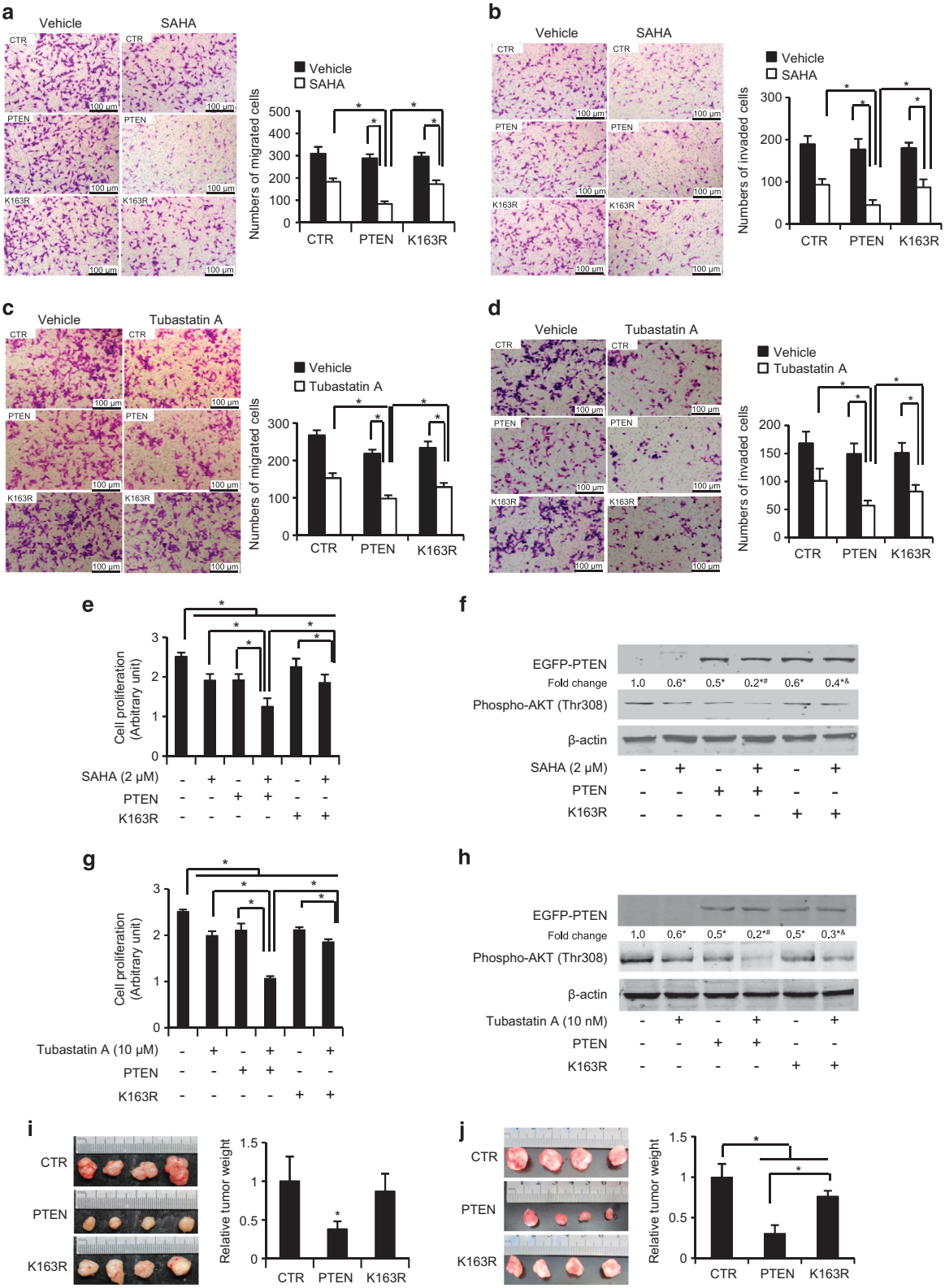


Figure 6. Inhibition of HDAC6 induced PTEN acetylation at K163 and membrane translocation. (**a**–**d**) Inhibition of HDAC6 by tubastatin A or tubacin induced the acetylation and membrane translocation of wild-type PTEN, but not K163R mutant. U-87 MG or NCI-H1650 cells were transfected with EGFP-PTEN or EGFP-PTEN-K163R for 24 h and then treated with tubastatin A or tubacin for 24 h. Experiments were performed as in Figures 2d and 3a. *$P < 0.01$. (**e**) Fluorescent microphotographs of 293T cells transfected with EGFP-PTEN or EGFP-PTEN-K163R and treated with tubastatin A for 24 h. (**f**, **g**) Knockdown of HDAC6 increased acetylation and membrane translocation of wild-type PTEN, but not K163R mutant. HDAC6 siRNA was co-transfected with EGFP-PTEN or EGFP-PTEN-K163R into U-87 MG or NCI-H1650 cells for 48 h. Experiments were performed as described above. *$P < 0.01$. IP, immunoprecipitation; WB, western blot.

Figure 7. Overexpression of wild-type PTEN, but not K163R mutant, facilitated SAHA- or tubastatin A-induced inhibition of cell migration, invasion and proliferation, as well as xenograft tumour growth, in U-87 MG cells. (**a**–**d**) Microphotographs of transwell migration or invasion of cells. Cells stably transfected with wild-type PTEN or PTEN-K163R mutant were treated or not treated with 2 μM SAHA or 4 nM tubastatin A. Transwell migration or invasion of the cells were photographed under a light microscope (left panel). Migrated cells were counted from six randomly selected fields (right panel). Bar = 50 μm. *$P < 0.05$ ($n = 6$). (**e**, **g**) SAHA-induced cell growth arrest. Cells stably transfected with wild-type PTEN or the PTEN-K163R mutant were treated with 2 μM SAHA or 10 nM tubastatin A for 72 h and subjected to CCK-8 assay. *$P < 0.05$. (**f**, **h**) SAHA- or tubastatin A-induced AKT dephosphorylation. Cells stably transfected with wild-type PTEN or PTEN-K163R mutant were treated with 2 μM SAHA or 10 nM tubastatin A for 72 h and subjected to western blot. *$P < 0.05$ versus the group of U-87 MG cells without PTEN transfection and SAHA treatment; $^{\#}P < 0.05$ versus all other groups; $^{\&}P < 0.05$ versus the group of U-87 MG cells with PTEN-K163R transfection and without SAHA treatment. (**i**, **j**) Photographs and weights of xenograft tumours. SAHA (**i**) and tubastatin A (**j**) inhibited xenograft tumour growth of cells stably transfected with either of the wild-type PTEN, PTEN-K163R mutant or empty vector in nude mice. *$P < 0.05$ ($n = 5$–6). CTR, control.

namely, TSA and SAHA, can induce the acetylation and membrane translocation of PTEN. As TSA and SAHA inhibit HDAC1–11 and NaBu inhibits HDAC1–9, except HDAC6,[22] only HDAC6, HDAC10 and HDAC11 may be involved in TSA- or SAHA-induced PTEN acetylation. As such, we excluded the involvement of HDAC10 and HDAC11 because their specific siRNAs did not affect PTEN acetylation and we speculated HDAC6 as a possible target for TSA or SAHA to increase PTEN acetylation. Hence, we performed a series of experiments to prove that HDAC6 regulated PTEN acetylation and membrane translocation or activation through PTEN acetylation at K163. We found that HDAC6-specific inhibitors, namely, tubastatin A or tubacin, or HDAC6-specific siRNA exhibited similar effects to those of TSA and SAHA on acetylation and membrane translocation of wild-type PTEN, but



not PTEN-K163R mutant; HDAC6 could interact with PTEN, and overexpression of HDAC6 inhibited acetylation and membrane translocation of PTEN. PTEN activation by HDAC6 inhibition was consistent with the results of an HDAC6 knockout study, in which AKT phosphorylation was downregulated.[27] On the basis of these findings, we conclude that non-selective HDACs inhibitors, namely, TSA or SAHA, induced acetylation and membrane translocation of PTEN through HDAC6 inhibition. Moreover, PTEN was revealed as a new target for HDAC6; as such, anti-tumour drugs targeting HDAC6 should be developed.[45]

Activation of PTEN by acetylation at K163 contributed to the anti-tumour effects of non-selective HDAC inhibitors or HDAC6-specific inhibitors. Non-selective HDAC inhibitors, such as SAHA, or HDAC6-specific inhibitors, such as tubastatin A, could more significantly inhibit cell proliferation, migration and invasion, as well as xenograft tumour growth, in PTEN-null U-87 MG cells stably transfected with wild-type PTEN, but not in PTEN-K163R mutant, than that in control U-87 MG cells. These results showed that ectopic PTEN facilitated the anti-tumour effects of SAHA or tubastatin A in PTEN-null U-87 MG cells. Hence, tumours with intact or mutated PTEN would have different responses to non-selective HDAC inhibitors or HDAC6-specific inhibitors. We also detected that SAHA or tubastatin A could inhibit cell proliferation, migration and invasion in U-87 MG cells or U-87 MG cells stably transfected with PTEN-K163R mutant. This finding could be because of other mechanisms underlying the anti-tumour effects of SAHA or tubastatin A. For instance, SAHA can induce the expression of p21Waf1/Cip1 to arrest cell cycle and promote apoptosis;[40] HDAC6 can inhibit AKT phosphorylation and cell proliferation by interacting with the protein phosphatase l;[46] and HDAC6 inhibition can also induce p53. Nevertheless, our results implied that the anti-tumour efficiency of non-selective HDAC inhibitors or HDAC6-specific inhibitors would be compromised in the presence of aberrant PTEN or PTEN deletion in the tumours. Therefore, application of non-selective HDAC inhibitors, such as SAHA, or HDAC6-specific inhibitors may be suitable for tumours with non-mutated or deleted PTEN. Otherwise, if the therapeutic window is insufficient, the use of these inhibitors may pose significant side effects.

In conclusion, we revealed that PTEN activation through K163 acetylation by HDAC6 inhibition significantly contributed to tumour inhibition. Application of non-selective HDAC inhibitors, such as SAHA, or HDAC6-specific inhibitors, may be suitable for tumours without PTEN mutation or deletion.

MATERIALS AND METHODS

Cell lines

293T cells derived from human embryonic kidney and PTEN-null U-87 MG cells derived from human glioma were incubated in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY, USA) with 10% fetal bovine serum at 37 °C under 5% CO_2. SACC-83 cells derived from human salivary adenoid cystic cancer and PTEN-null NCI-H1650 cells derived from non-small cell lung cancer were incubated in RPMI medium 1640 (GIBCO) with 10% fetal bovine serum at 37 °C under 5% CO_2.

Reagents and antibodies

TSA and NaBu were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tubastatin A, tubacin and SAHA were purchased from Selleck Chemicals (Houston, TX, USA).

Anti-PTEN antibodies (#9552), anti-phospho-AKT (Thr308) antibodies (#9275), anti-GFP antibodies (#2555), anti-acetylated-lysine rabbit monoclonal antibodies (Ac-K-103) (#9068), anti-acetylated-lysine mouse mAb (Ac-K-103) (#9681) and anti-HDAC6 antibodies (#7612) were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-β-actin (I-19) antibodies (sc-1616) were purchased from Santa Cruz Biotechology (Santa Cruz, CA, USA).

Plasmids and siRNAs

pcDNA3.1-HDAC6 plasmids (Addgene plasmid 13823, Cambridge, MA, USA) were kindly provided by Professor Verdin of the Gladstone Institute of Virology and Immunology, University of California, San Francisco.[47] The full length of human PTEN was amplified from Pbp-PTEN plasmids (provided by Professor Webster K. Cavenee of the Department of Medicine and Cancer Centre, University of California) with a high fidelity DNA polymerase (TOYOBO, Osaka, Japan) by using standard polymerase chain reaction (PCR) techniques. The amplified gene was cloned into pZeroBack/blunt vectors (Tiangen, Beijing, China) and recloned into EGFP-C1 plasmids at *Hin*dIII and *Eco*RI sites. Primers with restriction enzyme sites (underlined) for cloning PTEN were commercially synthesised by Shanghai Sangon Biotech (Shanghai, China) based on the following sequences: 5′-AAGCTTATGACAGCCATCATCAAAGAGA-3′ (sense) and 5′-GAATTCGACTTTTGTAATTTGTGT-3′ (antisense). A series of deletion mutants of EGFP-PTEN were constructed based on the full-length PTEN construct. All constructs were confirmed through DNA sequencing.

Deletion mutagenesis

Deletion mutagenesis was performed as described previously[42] by using EGFP-PTEN plasmid as the template with primers designed surrounding the deleted region. The following primers for EGFP-PTEN-ΔC were used: 5′-GAATTCTGCAGTCGACGGTAC-3′ (sense) and 5′-CTCTACTGTTTTTGTGAAGTAC-3′ (antisense). For EGFP-PTEN-ΔP, the following primers were used: 5′-CTGGATTATAGACCAGTGGC-3′ (sense) and 5′-AAGCTTGAGCTCGGAGATCTG-3′ (antisense). Finally, the primers for EGFP-PTEN-P included the following: 5′-GAATTCTGCAGTCGACGGTAC-3′ (sense) and 5′-ATGATTCTTTAACAGGTAGC-3′ (antisense). All primers were commercially synthesised by Shanghai Sangon Biotech. All constructs were confirmed by DNA sequencing (the sequences could be obtained at http://dx.doi.org/10.6084/m9.figshare.1441174).

Protein extraction and western blot analysis

Whole-cell lysates were extracted using RIPA lysis buffer (Applygen, Beijing, China). Membrane proteins were extracted using Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA). Protein concentrations were determined through BCA protein assay (Thermo Fisher Scientific Inc.). Equal amounts of samples were subjected to 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% fat-free milk in TBS-T (50 mmol/l Tris, pH 7.5; 150 mmol/l NaCl; 0.05% Tween 20) for 1 h. After incubation with primary antibodies diluted at 1:1000 in TBS-T containing 1% milk overnight at 4 °C, the membrane was washed extensively with TBS-T and then incubated with a secondary antibody conjugated with fluorophore for 1 h at room temperature. After extensive washing with TBS-T, the membrane was visualised using the Odyssey infrared imaging system (Odyssey LI-COR, Lincoln, NE, USA). For internal controls of equal loading, the blots were placed in stripping buffer (100 mmol/l 2-mercaptoethanol; 2% sodium dodecyl sulphate; 62.5 mmol/l Tris, pH 6.8) and reprobed with β-actin antibody.

Real-time quantitative PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription and real-time PCR were performed as described previously.[41] The primers used for human HDAC10 were previously reported:[48] 5′-ATCTCTTTGAGGATGACCCCAG-3′ (sense) and 5′-ACTGCGTCTGCATCTGACTCTC-3′ (antisense). The primers for human HDAC11 were designed using the Primer Premier Version 5.0 as follows: 5′-TCTACAACCGCCACATCTACCCA-3′ (sense) and 5′- TGCGGAGACGCTGGGTGA-3′ (antisense). The efficiency of the primers was confirmed by sequencing their conventional PCR products. Real-time PCR was performed in a 7500 real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) with FastStart Universal SYBR Green Master Roche (Basel, Swiss).

Confocal fluorescence microscopy

293T cells were transfected with EGFP-PTEN or EGFP-PTEN-K163R for 24 h in the presence or absence of TSA, SAHA or tubastatin A. The cells were washed in phosphate-buffered saline three times, fixed with 95% ethanol and washed again in phosphate-buffered saline three times. The location of EGFP-PTEN was visualised using a Zeiss (Oberkochen, Germany) laser

scanning microscope (LSM 510) at the wavelength of 488nm. Images were processed using LSM 5 Release 4.2 software.

Site-directed mutagenesis

Site-directed mutagenesis was performed as described previously.[42] Briefly, PCR was performed using a high-fidelity DNA polymerase (TOYOBO) with cycles at 16–18 and the PCR products were treated with 1 μl of Dpn I at 37 °C for 1–2 h to digest parental DNA. All primers were commercially synthesised (Sangon Biotech Co., Ltd., Shanghai, China). The primers used for K163 mutation of PTEN to arginine included the following: 5′-CCAGAGACAGAAAGGGAGTAACTA-3′ (sense) and 5′-TAGTTACTCCCTTTC TGTCTCTGG-3′ (antisense), where the underlined sequences represent the mutated nucleotides. Mutation was confirmed by DNA sequencing (the sequences could be obtained at http://dx.doi.org/10.6084/m9.figshare.1441174).

Immunoprecipitation

The cells were washed three times with ice-cold phosphate-buffered saline and placed in non-denaturing lysis buffer (Applygen Technologies, Inc., Beijing, China). Equal amounts (500 μg) of whole-cell lysates were incubated with 4 μg of anti-EGFP, anti-PTEN or anti-Flag antibodies for 16 h with rotation of 5000 *g* at 4 °C. After addition of 40 μl of protein A/G-agarose beads (Santa Cruz Biotechnology), the mixture was further incubated with rotation for 1 h at 4 °C and then centrifuged (12000 *g* for 1 min). Resultant pellets were washed five times. The bound proteins were released by boiling in 30 μl of sodium dodecyl sulphate loading buffer and then subjected to western blot analysis. Acetylated PTEN was detected using anti-acetyl lysine antibodies. The membranes were stripped and reprobed with anti-PTEN antibodies.

Stable transfection with lentivirus

Full-length human wild-type PTEN and K163R mutant were amplified from EGFP-PTEN and EGFP-K163R by using standard PCR techniques, cloned into pZeroback/blunt vectors (Tiangen, Beijing, China) and recloned into pLVX-AcGFP-N_1 vectors (Clontech, Mountain View, CA, USA). The constructs were confirmed by DNA sequencing. The plasmids pLVX-AcGFP-N_1-PTEN, pLVX-AcGFP-N_1-PTEN-K163R and pLVX-AcGFP-N_1 were each co-transfected into 293T cells by using Lenti-X HTX Packaging Mix (Clontech). Lentiviral supernatants were collected 48 h after transfection and then centrifuged (500 *g* for 10 min at 4 °C). The supernatant was added to U-87 MG cells, which were subsequently cultured for 72 h. The infected U-87 MG cells were screened using 1 μg/ml puromycin, and over-expression of PTEN and its mutant in U-87 MG cells were confirmed with western blot assay.

Cell proliferation assay

Cell proliferation assay was performed using Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, the cells were seeded into 96-well plates (1.5×10^3 cells per well) and treated with 2 μM SAHA or 10 μM tubastatin A. At 72 h after treatment, 10 μl of CCK-8 was added to each well and incubated at 37 °C for 3 h. Absorbance at 450 nm was determined.

Transwell migration and invasion assay

Cell migration and invasion assays were performed in transwell chambers (Corning Costar, Corning, NY, USA) by using a polycarbonate membrane as described previously.[42] Briefly, for migration assays, the cells were seeded at 10^5 cells per well in serum-free medium in the upper chambers, and the lower chambers contained the culture medium with 10% fetal bovine serum. The cells were then incubated for 12 h. Cells on the top surface of the membrane were wiped off, whereas those on the bottom surface were fixed with 4% paraformaldehyde and stained with 0.01% crystal violet. Cells on the bottom surface of the membrane were examined under a light microscope, counted and averaged by the number of six randomly selected fields. The same procedure was performed for transwell invasion assay, except that the upper chambers were coated with 20 μg of extracellular matrix gel prior to seeding of the cells (Sigma-Aldrich).

Xenograft tumour inoculation

BALB/c nude mice (5 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). The care and treatment of experimental animals followed the institutional guidelines. Mice were randomly allocated to each group ($n = 8$). U-87 MG cells stably transfected with empty vectors or wild-type PTEN or mutant PTEN-K163R were subcutaneously inoculated (5×10^6 cells/mouse) in the right flanks of mice ($n = 8$). After 10 days, mice of each group received 50 mg/kg per day SAHA or 0.5 mg/kg per day tubastatin A (both dissolved in dimethyl sulphoxide) through intraperitoneal injection daily for 3 weeks. The mice were killed, and the weights of xenograft tumours were measured.

Statistical analysis

Statistical analysis was performed using SPSS 11.5 for Windows. All experiments were repeated three times and all data were presented as mean ± standard deviation. The differences between multiple groups were analysed by one-way analysis of variance. $P < 0.05$ was considered as statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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