Histone deacetylase 1 and 2 differentially regulate apoptosis by opposing effects on extracellular signal-regulated kinase 1/2

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Histone deacetylases (HDACs) are epigenetic regulators that are important for the control of various pathophysiological events. We found that HDAC inhibitors completely abolished transforming growth factor-β1 (TGF-β1)-induced apoptosis in AML-12 and primary mouse hepatocytes. Expression of a dominant-negative mutant of HDAC1 or downregulation of HDAC1 by RNAi both suppressed TGF-β1-induced apoptosis. In addition, overexpression of HDAC1 enhanced TGF-β1-induced apoptosis, and the rescue of HDAC1 expression in HDAC1 RNAi cells restored the apoptotic response of cells to TGF-β1. These data indicate that HDAC1 functions as a proapoptotic factor in TGF-β1-induced apoptosis. In contrast, downregulation of HDAC2 by RNAi increased spontaneous apoptosis and markedly enhanced TGF-β1-induced apoptosis, suggesting that HDAC2 has a reciprocal role in controlling cell survival. Furthermore, inhibition of extracellular signal-regulated kinase 1/2 (ERK1/2) by MEK1 inhibitor PD98059 or expression of a kinase-dead mutant of MEK1 restored the apoptotic response to TGF-β1 in HDAC1 RNAi cells. Strikingly, HDAC2 RNAi caused an inhibition of ERK1/2, and the spontaneous apoptosis can be abolished by reactivation of ERK1/2. Taken together, our data demonstrate that HDAC1 and 2 reciprocally affect cell viability by differential regulation of ERK1/2; these observations provide insight into the roles and potential mechanisms of HDAC1 and 2 in apoptosis.

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Apoptosis is a highly controlled process that has been implicated in various physiological and pathological events. During apoptosis, cells shrink, chromatin becomes fragmented, the nucleus becomes condensed and/or fragmented. Disruption of the normal control of apoptosis has been linked with various pathological events. Transforming growth factor (TGF)-β is a pleiotropic growth factor that positively or negatively regulates many biological events depending on cell status, cell type and environmental context. TGF-β has been shown to be a strong inducer of apoptosis in primary rat hepatocytes, Hep-3B, HuH-7, FaO hepatoma cell lines and AML-12 murine hepatocytes.1–6 In addition, dysregulation of TGF-β-mediated apoptosis occurs in many diseases, such as fibrosis, autoimmunity, inflammation and tumorigenesis.7–9 Although tremendous progress has been made in exploring the regulatory effects of TGF-β1-induced apoptosis, further studies are important for a better understanding of the underlying mechanisms.

Mitogen-activated protein kinase (MAPK) cascades are conserved signal-transduction pathways that respond sensitively to diverse extracellular stimuli in mediating cell proliferation, differentiation, migration, stress responses, inflammation and apoptosis.10 Extracellular signal-regulated kinases 1/2 (ERK1/2) are important survival factors that have been shown to have significant roles during TGF-β-mediated biological events. ERK1/2 inactivation has been reported to enhance TGF-β-induced apoptosis and the suppression of proliferation;11–14 whereas ERK1/2 activation is involved in TGF-β-induced suppression of apoptosis and promotion of proliferation.15–17 ERK1/2 are also required for TGF-β-induced cell migration18–20 and the epithelial-to-mesenchymal transition.21,22 However, knowledge regarding the factors that control ERK1/2-mediated cell viability still remains limited.

Not only do histone deacetylases (HDACs) classically function as epigenetic regulators through histone deacetylation but they can also function on other proteins such as transcription factors to regulate gene transcription. HDACs control various biological events including cardiac development, skeletogenesis, cytoskeletal dynamics and the integrity of endothelium.23 There are 18 mammalian HDACs, which

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Abbreviations: HDAC, histone deacetylase; TGF-β1, transforming growth factor-β1; ERK1/2, extracellular signal-regulated kinase 1/2; MEK1, MAPKK1, mitogen-activated protein kinase kinase 1; DN-HDAC1, dominant-negative HDAC1; KD-MEK1, kinase-dead MEK1; TSA, trichostatin A; NaBu, sodium butyrate; AO/EB, acridine orange/ethidium bromide; MDCK, Madin–Darby canine kidney; EGF, epithelial growth factor

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are grouped into four classes. The class I HDACs include HDAC1, 2, 3 and 8 and are predominantly localised in the nucleus. HDAC1 and 2 are the two closest homologues, sharing the highest level of sequence identity. They hetero-
dimerise and form complexes with other molecules to execute the same functions, such as the regulation of cardiac development.24 HDAC1 and 2 have also been shown to have different biological functions. During embryonic differentia-
tion, HDAC1 is required for neuronal differentiation, whereas HDAC2 specifically inhibits astrocyte differentiation.25 There are also discrepancies between the reported roles of HDAC1 and 2 in apoptosis. Knockdown of HDAC1 but not HDAC2 in U2OS osteosarcoma cells has been shown to induce apoptosis,26 whereas the knockdown of HDAC2 but not HDAC1 in HeLa cells results in apoptosis.27 However, there is no evidence regarding the roles of HDAC1 and 2 in controlling apoptosis in normal cells.

HDAC inhibitors are potent inducers of apoptosis in cancer cells. They suppress tumour growth and progression, induce the differentiation of transformed cells and inhibit tumour angio genesis and invasion in vivo.28 Hence, HDAC inhibitors have been considered as potential drugs for cancer treatment. HDAC inhibitors are also well-studied for their selectivity, because they can induce the apoptosis of cancer cells but possess less toxicity towards non-cancer cells.29 Death receptor and mitochondrial death pathways have been shown to participate in HDAC inhibitor-induced apoptosis.30 However, very little is known about the biological effects of HDAC inhibitors on the viability of normal cells. In addition, most HDAC inhibitors inhibit HDACs non-selectively, so the roles of different HDACs cannot be identified using HDAC inhibitors alone.

In this study, we examined the roles of HDAC inhibitors, HDAC1 and 2, on TGF-β1-induced apoptosis in mouse hepatocytes. We showed that HDAC inhibitors suppress TGF-β1-induced apoptosis and demonstrated that HDAC1 and 2 had opposite roles in apoptosis. The study revealed that opposing regulation of ERK1/2 contributed to the opposite roles of HDAC1 and 2.

Results

HDAC inhibitors suppress TGF-β1-induced apoptosis in hepatocytes. To investigate whether HDACs, which are major epigenetic regulators, are involved in the control of cell apoptosis, we examined the effects of three structurally unrelated HDAC inhibitors, trichostatin A (TSA), sodium butyrate (NaBu) and MS-275, on TGF-β1-induced apoptosis in AML-12 hepatocytes. AML-12 cell line was established from hepatocytes from the mouse transgenic for human TGF-α.31 These cells exhibit typical hepatocyte features, such as peroxisomes and bile canalicular-like structure and expressing high levels of serum and gap junction proteins.31 Most importantly, AML-12 cells are as sensitive as primary hepatocytes to the TGF-β1, and results obtained from AML-12 cells are highly consistent with that from primary hepatocytes. TGF-β1 treatment induces both apoptosis and epithelial–mesenchymal transition.32,33 As shown in Figure 1, HDAC inhibitors inhibited TGF-β1-induced apoptosis, as determined by FACS (Figure 1a), acridine orange/ethidium bromide (AO/EB) staining assay (Figure 1b), caspase-3 activity (Figure 1c) and DNA fragmentation (Supplementary Figure 1a–c). Consistently, all three HDAC inhibitors also abolished TGF-β1-induced apoptosis in primary mouse hepatocytes, as determined by FACS (Figure 1d), AO/EB staining (Figure 1e) and caspase-3 activity (Figure 1f). The above results suggest that HDACs have critical roles in TGF-β1-induced apoptosis in hepatocytes. These results indicate that HDAC1 may be involved in TGF-β1-induced apoptosis in hepatocytes. Because MS-275 is a relative HDAC1-selective inhibitor, whose IC50 value for HDAC134,35 is much lower than that for other HDACs, and there is an increase in the HDAC1 but not HDAC2 activity during TGF-
β1-induced apoptosis in AML-12 (Supplementary Figure 2a) and primary hepatocytes (Supplementary Figure 2b), the effect of MS-275 may be linked with the inhibition of HDAC1 activity.

HDAC1 is required for TGF-β1-induced apoptosis. Next we investigated the roles of HDAC1 in TGF-β1-induced apoptosis. We constructed dominant-negative c-myc-tagged HDAC1 (DN-HDAC1) plasmids by substituting a conserved histidine (histidine 141) in the catalytic domain with an alanine.36,37 Stably transfected cell clones were obtained by transfection and were examined by immunoblotting (Supplementary Figure 3a). The results show that TGF-β1-
induced apoptosis was completely suppressed in DN-HDAC1 cells, as detected by FACS (Figure 2a) and AO/EB staining (Figure 2b). To confirm the role of HDAC1, GFP-tagged wild-type HDAC1 or GFP empty vector plasmid was transfected into AML-12 cells and stable clones were selected by detecting GFP: The HDAC1–GFP fusion proteins were exclusively localised in the nucleus, compared with the diffuse distribution pattern of soluble GFP (Supplementary Figure 3b). Both GFP and HDAC1–GFP can also be detected by immunoblotting with GFP antibody (Supplementary Figure 3c). Compared with overexpression of GFP alone, overexpression of HDAC1–GFP significantly increased TGF-β1-induced apoptosis (Figure 2c). To further confirm the above results, we constructed two HDAC1 RNAi plasmids with different target sequences (iHDAC1-S1 and -S2). After transfection, stable cell clones were selected (Supplementary Figure 3d). As determined by FACS, TGF-β1-induced apoptosis was significantly decreased in iHDAC1 cells (Figure 2d). The inhibition of TGF-β1-induced apoptosis by HDAC1 RNAi was also identified by DNA fragmentation (Figure 2e) and AO/EB staining (Figure 2f) assays. In addition, reintroduction of RNAi-resistant human GFP-tagged HDAC1 back into iHDAC1 cells restored the TGF-β1-induced apoptotic response (Figure 2g). These data demonstrate that HDAC1 is required for TGF-β1-induced apoptosis in AML12 mouse hepatocytes.

HDAC2 is required for survival of AML-12 hepatocytes. To study the role of HDAC2 in TGF-β1-
induced apoptosis, HDAC2 RNAi plasmid (iHDAC2) was constructed and transfected into AML-12 cells. Several stable cell clones were selected (Supplementary Figure 3d). Surprisingly, downregulation of HDAC2 by
HDAC2 RNAi caused spontaneous cell apoptosis, as determined by FACS (Figure 3a), DNA fragmentation (Figure 3b) and AO/EB staining (Figure 3c). In addition, downregulation of HDAC2 significantly increased the apoptotic response of cells to TGF-β1 treatment (Figure 3d–f). These results indicate that in contrast to HDAC1, HDAC2 has important part in the survival of AML-12 hepatocytes.

**HDAC inhibitors abolish TGF-β1-induced inhibition of ERK1/2 to suppress apoptosis.** ERK1/2 is a well-known survival factor and its inactivation has been recently shown to increase the TGF-β1-induced apoptosis.12 TGF-β1 treatment inhibited ERK1/2 as shown by a decrease in the levels of phosphorylated ERK1/2 (p-ERK1/2) in AML-12 cells and primary hepatocytes (Figure 4a). Interestingly, TGF-β1 did not inhibit ERK1/2 in MDCK (Madin–Darby canine kidney), 3T3-L1 and A549 cells (Supplementary Figure 4a), which corresponds to its inability to induce apoptosis in these cells (Supplementary Figure 4b). Thus, it suggests that inhibition of p-ERK1/2 contributed to TGF-β1-induced apoptosis. Moreover, TSA treatment activated ERK1/2 in AML-12 and primary hepatocytes in time- and dose-dependent manners (Figure 4b). Similar effect of NaBu and MS-275 was also observed in AML-12 cells (Supplementary Figure 5a and b). Consistent with these observations, TSA treatment partially but significantly reduced TGF-β1-induced inhibition of ERK1/2 levels (Figure 4c). In the presence of PD98059, an inhibitor of mitogen-activated protein kinase kinase 1 (MAPKK1, MEK1), the basal level of activated ERK1/2 was significantly reduced, and more pronounced decrease was observed in the presence of both PD98059 and TGF-β1.
Correspondingly, much stronger apoptosis was induced by TGF-β1 and PD98059 used together (Figure 4e). However, because TSA can fundamentally induce the ERK1/2 activation even in the presence of both PD98059 and TGF-β1, apoptosis induced by TGF-β1 and PD98059 used together was still markedly reduced by TSA in AML-12 and primary mouse hepatocytes (Figure 4e). These results suggest that ERK1/2 activation is required for HDAC inhibitor-mediated suppression of TGF-β1-induced apoptosis in hepatocytes.

**HDAC1 regulates apoptosis by inhibiting ERK1/2 phosphorylation.** To determine whether the regulation of ERK1/2 is related to the role of HDAC1 in TGF-β1-mediated apoptosis, we examined p-ERK1/2 levels in HDAC1 small interfering RNA-transfected cells. In iHDAC1 cells, as compared with empty vector-transfected cells, TGF-β1 treatment cannot inhibit the ERK1/2 (Figure 5a). Furthermore, inhibition of ERK1/2 by PD98059 restored the apoptotic response of iHDAC1 cells to TGF-β1 stimulation (Figure 5b), suggesting further that HDAC1 mediates TGF-β1-induced apoptosis by inactivating ERK1/2. To confirm these results, AML-12 cells were transfected with the kinase-dead form (K97A) of MEK1 (KD-MEK1). As a result, the p-ERK1/2 levels in cells stably transfected with the HA-tagged KD-MEK1 were significantly reduced (Supplementary Figure 6a) and the apoptotic response of cells to TGF-β1 was enhanced (Supplementary Figure 6b). Interestingly, TSA
treatment restored p-ERK1/2 to background levels (Supplementary Figure 6c) and also reduced TGF-β-induced apoptosis in KD-MEK1 cells (Supplementary Figure 6d). In addition, overexpression of GFP-tagged KD-MEK1 in iHDAC1 cells (Figure 5c) restored the apoptotic response of these cells to TGF-β1 (Figure 5d). These results further support that p-ERK1/2 has a critical role in HDAC1-mediated TGF-β1-induced apoptosis in AML-12 mouse hepatocytes.

HDAC2 increases cell viability through activation of ERK1/2. Because HDAC2 downregulation increased apoptotic response to TGF-β1 (Figure 3) and caused significant spontaneous apoptosis (Figure 3), HDAC2 appeared important for the survival of AML-12 hepatocytes. Indeed, ERK1/2 were significantly inhibited in iHDAC2 cells compared with control and iHDAC1 cells (Figure 6a). Treatment of cells with epithelial growth factor (EGF), which activated ERK1/2 in iHDAC2 cells (Figure 6b), reduced the spontaneous apoptosis (Figure 6c), indicating that ERK1/2 inhibition contributed at least in part to the spontaneous apoptosis in iHDAC2 cells. In addition, TSA treatment also restored the levels of activated ERK1/2 levels in iHDAC2 cells (Figure 6d) and suppressed spontaneous apoptosis (Figure 6e). Inhibition of HDAC1 by MS-275 also activated ERK1/2 (Figure 6f) and inhibited apoptosis in iHDAC2 cells (Figure 6g), suggesting that HDAC1 functions as a proapoptotic factor by inhibiting ERK1/2 during spontaneous apoptosis in HDAC2-deficient cells.

Discussion

HDAC inhibitors have been widely used to investigate the roles and involvement of HDACs in various biological events. In this study, we showed that HDAC inhibitors powerfully suppress TGF-β1-induced apoptosis in mouse hepatocytes.
This suppression of TGF-β1-induced apoptosis is a new function of HDAC inhibitors that has not been investigated and demonstrated in previous studies. In addition, our results demonstrate that three different HDAC inhibitors activate ERK1/2 to suppress TGF-β1-induced apoptosis. Our results are consistent with the previous reports that the HDAC inhibitor valproic acid can activate ERK1/2, resulting in suppression of serum starvation-induced apoptosis in human umbilical vein endothelial cells. Another HDAC inhibitor, apicidin, was also shown to activate ERK1/2. All of these HDAC inhibitors are structurally unrelated: TSA is a hydroxamic acid compound, NaBu and valproic acid are short-chain fatty acids, whereas MS-275 is a benzamide and apicidin is a cyclic tetrapeptide. This indicates that activation of ERK1/2 is a common event induced by HDAC inhibitors and suggests that modulation of ERK1/2 activity is an important function of HDACs. By immunofluorescence staining and immunoblotting, we found that HDAC inhibitors have no effect on the subcellular localisation and the expression levels of HDACs, suggesting that the regulation of subcellular localisation and
the expression of HDACs are not involved in the antiapoptotic function of HDAC inhibitors (data not shown).

Further study revealed that HDAC1 serves as a proapoptotic factor and HDAC2 serves as an antiapoptotic factor during TGF-β1-induced apoptosis in AML-12 hepatocytes. These findings suggest that HDAC1 and 2 possess reciprocal functions in the TGF-β signalling pathway during apoptosis. Roles of HDACs in TGF-β1-mediated biological events have begun to emerge in the past several years. It has been recently reported that HDAC2 functions as a key regulator for TGF-β1-induced renal injury.40 HDAC4 reportedly acts as a regulator of TGF-β1-induced myofibroblastic differentiation.41 HDAC6 has been shown to be required for TGF-β1-mediated epithelial–mesenchymal transition.42 HDAC4 and 5 were shown to be recruited by Smad3 to repress the transcription of Runx2 during TGF-β1-mediated inhibition of osteoblast differentiation.43 To the best of our knowledge, it is not known whether HDACs are involved in the regulation of TGF-β1-mediated apoptosis. This study provides the first insight into the roles and the potential mechanisms of HDAC1 and 2 in TGF-β1-induced cell apoptosis.

ERK1/2 are important survival factors that promote cell growth and inhibit apoptosis.44,45 Our results showed that inactivation of ERK1/2 occurs during TGF-β1-induced apoptosis. In iHDAC1 cells that do not undergo apoptosis by TGF-β1 treatment, p-ERK1/2 levels remained high in response to TGF-β1, whereas in iHDAC2 cells, the basal level of p-ERK1/2 was low, consistent with enhancement of spontaneous and TGF-β1-induced apoptosis. These results confirm that activation of ERK1/2 corresponds to the viability of AML-12 hepatocytes. Ramesh et al.46 reported that inhibition of ERK1/2 through the activation of MAPK phosphatase MKP2 is involved in the TGF-β1-induced expression of the proapoptotic protein BIM and in apoptosis. This study demonstrates that reverse regulation of ERK1/2 is related to opposing roles of HDAC1 and 2 in apoptosis.

Lagger et al.47 reported that expression of HDAC2 and 3 was induced in HDAC1-deficient cells but could not compensate for the loss of HDAC1. In this study, the protein levels of HDAC2 and 1 were also reciprocally upregulated in iHDAC1 and 2 cells. However, it is not clear whether such increases have a respective role in these cells.

Because most HDAC inhibitors do not show specificity to different HDACs, the general or net effect of HDAC inhibitors cannot reflect the distinct roles of different HDACs. Our results demonstrate that HDAC1 and 2 have opposing roles in apoptosis. However, HDAC inhibitors, which can suppress both HDAC1 and 2, were found to inhibit apoptosis. These results indicate that suppression of HDAC1 but not HDAC2 can account for the inhibitory effect of HDAC inhibitors.
inhibitors during apoptosis. In addition, we also found that HDAC inhibitors suppressed spontaneous apoptosis in HDAC2-deficient cells, suggesting that the suppression of apoptosis by HDAC inhibitors is independent of HDAC2. As HDAC inhibitors has been reported to induce strong apoptosis in cancer cells, our finding that HDAC inhibitors can suppress apoptosis in non-cancer cells also suggests distinct physiological contexts between cancer and non-cancer cells.

In conclusion, our data demonstrate that HDAC1 and 2 have opposing roles during TGF-β1-induced apoptosis in AML-12 hepatocytes: the former functions as a proapoptotic factor, whereas the latter functions as a survival factor. These roles are dependent on opposing effects on ERK1/2 activation. This study provides insight into the role and potential mechanism of HDAC1 and 2 on cell survival and apoptosis. As a summary of our study, a schematic illustration is shown in Figure 7.
Regulation of cell apoptosis by HDAC1 and 2

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Materials and Methods

Materials. AML-12, MDCK and A549 cells were originally purchased from American Type Culture Collection (Manassas, VA, USA). TSA, NaBu, MS-275, Ac-DEVD-pNA (caspase-3 substrate) and human recombinant EGF were purchased from Sigma-Aldrich (St. Louis, MO, USA). P98059 was from Calbiochem (La Jolla, CA, USA). Human recombinant TGF-β1 was from Chemicon (Rosemont, IL, USA). Antibodies for β-actin, c-myc, HA, phospho-ERK1/2, ERK2 and HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Preparing HDAC1 and 2 anti-serum. The sequence differences between HDAC1 and 2 only exist at their C-terminals, so plasmodalms containing the C-terminal sequences of HDAC1 and 2 in pGEX-4T-1 vector (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) were constructed. The primer sequences used were as follows: 5'-GGAGAAGGTTGCTCCGAGAACCTTGTC-3' and 5'-CTGCGAGATCTGGCTCAGGCC-3' for HDAC1, and 5'-CTGCGAGATCTGGCTCAGGCC-3' and 5'-CTGCGAGATCTGGCTCAGGCC-3' for HDAC2. The GST-tagged HDAC1 and 2 proteins were expressed and purified for immunisation of New Zealand white rabbits. The serum was collected after three immunisations.

Cell culture. Cell culture and transfection reagents were from Invitrogen (Carlsbad, CA, USA). AML-12 murine hepatic epithelial cells were cultured in 1:1 mixture of Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F12 containing 10% fetal bovine serum and supplied with penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were incubated at 37°C in a humidified atmosphere with 5% CO2. Experiments were performed when cells reached 40–60% confluence.

Preparation of cell lysates and immunoblotting. Cells were lysed in lysis buffer containing 50 mmol/l HEPES (pH 7.4), 5 mmol/l EDTA, 5 mmol/l NaCl, 1% Triton X-100, 50 mmol/l NaF, 10 mmol/l Na3P2O7, 100 μl/ml, 5 μg/ml apritolin, 5 μg/ml leupeptin, 1 mmol/l Na3VO4, and 1 mmol/l phenylmethylsulphonyl fluoride. Proteins (30 μg) were electrophoresed in SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. The membranes were subsequently blocked with 5% skimmed milk and incubated with appropriate primary and secondary antibodies. Protein bands were visualised with super signal reagents.

Caspase-3 activity assay. Cells in 35-mm dishes were harvested by trypsin digestion and then were lysed in lysis buffer (containing 50 mmol/l HEPES (pH 7.4), 5 mmol/l CHAPS and 5 mmol/l DT) on ice for 20 min. After centrifuging, 50 μl supernatant was mixed with 50 μl assay buffer (containing 0.4 mmol/l Ac-DEVD-pNA, 4 mmol/l EDTA and 5 mmol/l DT). Absorbance at 405 nm was measured after the mixtures were incubated at 37°C for 8 h. For statistical analysis of several experiments, the negative controls were set as 1.0.

HDAC activity assay. Cells in 35-mm dishes were lysed in the same lysis buffer as an immunoblotting assay. After centrifuging, the supernatant was immunoprecipitated with 1 μl of anti-serum of HDAC1 (or 2) and 20 μl of slurry of 50% protein A Sepharose CL-4B beads (GE Healthcare, Piscataway, NJ, USA) on a rotator at 4°C overnight. The immunoprecipitated beads were washed with lysis buffer and PBS three times, respectively. Then HDAC1 or 2 activity was examined by HDAC assay kit from Upstate (Milton, Billerica, MA, USA). Absorbance at 405 nm was calculated and for statistic data of several experiments, the negative controls were set as 1.0.

Cell transfection. Cells in 35-mm dishes were transfeeted with 2 μg of plasmid using the Lipofectamine transfection reagent (Invitrogen) according to the manufacturer’s instruction. For transient transfection, expression of the indicated plasmids was examined 48 h after transfection. For selecting the stable cell clones, G418 (800 μg/ml) was added 48 h after the transfection.

Confocal analysis. Cells stably transfected with GFP and HDAC1–GFP were grown on glass slides. Confocal images were obtained using a confocal microscope. The fluorescence was visualised using confocal fluorescence microscopy (Leica, Mannheim, Germany).

Statistical analysis. Quantitative data were expressed as means ± S.D. or S.E. for at least three independent experiments. Statistical significance was determined by Student’s t-test(3,8),(997,997)
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