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Tubulin Beta3 Serves as a Target of HDAC3 and Mediates Resistance to Microtubule-Targeting Drugs

Youngmi Kim[1,2], Hyuna Kim[1,2], and Dooil Jeoung[1,*]

We investigated the role of HDAC3 in anti-cancer drug-resistance. The expression of HDAC3 was decreased in cancer cell lines resistant to anti-cancer drugs such as celastrol and taxol. HDAC3 conferred sensitivity to these anti-cancer drugs. HDAC3 activity was necessary for conferring sensitivity to these anti-cancer drugs. The down-regulation of HDAC3 increased the expression of MDR1 and conferred resistance to anti-cancer drugs. The expression of tubulin β3 was increased in drug-resistant cancer cell lines. ChIP assays showed the binding of HDAC3 to the promoter sequences of tubulin β3 and HDAC6. HDAC6 showed an interaction with tubulin β3. HDAC3 had a negative regulatory role in the expression of tubulin β3 and HDAC6. The down-regulation of HDAC6 decreased the expression of MDR1 and tubulin β3, but did not affect HDAC3 expression. The down-regulation of HDAC6 conferred sensitivity to taxol. The down-regulation of tubulin β3 did not affect the expression of HDAC6 or MDR1. The down-regulation of tubulin β3 conferred sensitivity to anti-cancer drugs. Our results showed that tubulin β3 serves as a downstream target of HDAC3 and mediates resistance to microtubule-targeting drugs. Thus, the HDAC3-HDAC6-Tubulin β axis can be employed for the development of anti-cancer drugs.

INTRODUCTION

Among the numerous HDACs, histone deactylase-3 (HDAC3) is ubiquitously expressed and conserved in a wide range of species (Mahlknecht et al., 1999). HDAC3 forms large corepressor complexes containing N-CoR/SMRT and additional proteins (Li et al., 2000). HDAC3 regulates the JNK pathway (Zhang et al., 2002), NF-κB activity (Chen et al., 2001), and MAPK activation (Mahlknecht et al., 2004). HDAC3 represses CREB3-mediated transcription and migration of metastatic breast cancer cells (Kim et al., 2010a). It localizes to the mitotic spindle and is required for kinetochore-microtubule attachment (Ishii et al., 2008). Aurora kinase B plays a critical role in mitosis. Aurora kinase B activity is required for mitotic processes, including kinetochore-microtubule attachment and chromosome congression (Fadri-Moskwik et al., 2012). Aurora kinase B activity is regulated by histone acetylation/deacetylation (Fadri-Moskwik et al., 2012). HDAC3 transiently interacts with Aurora kinase B and leads to reduction of acetylation of Aurora kinase (Fadri-Moskwik et al., 2012). Aurora kinase B is active in its deacetylated state (Fadri-Moskwik et al., 2012). Over-expression of Aurora kinase has been reported in multiple tumors and the selective inhibition of Aurora kinase B results in apoptosis induction (Xie et al., 2013). Aurora kinase B is regulated by the MAPK/ERK pathways and is a potential target for overcoming resistance to vemurafenib in metastatic melanomas (Bonet et al., 2012)

Phase I trial revealed that albumin-bound paclitaxel shows encouraging activity against advanced metastatic melanomas (Ott et al., 2013). Resistance to taxol, a microtubule-targeting drug, in hepatoma cells is related to JNK activation and prohibition into mitosis (Chae et al., 2012). Taxol-resistance results from MAPK activation (Xu et al., 2011). The inhibition of MAPK enhances taxol-induced apoptosis (Xu et al., 2009). As HDAC3 suppresses JNK (Zhang et al., 2002) and MAPK activation (Mahlknecht et al., 2004), it is likely that HDAC3 may regulate taxol-resistance.

The increased expression level of tubulin β3 is closely related with resistance to taxol (Kavallaris et al., 1997). High tubulin β3 expression is closely related with non-responsiveness to chemotherapy and is regulated by multiple signaling pathways, including PI3 kinase/Akt, Ras and MAP-ERK kinase (Levallet et al., 2012). HDAC6 deacetylates alpha-tubulin and regulates microtubule-dependent cell motility (Hubbert et al., 2002). This suggests that histone acetylation/deacetylation may also regulate activity and/or expression of tubulins. These reports suggested potential role of HDAC3 in resistance to microtubule-targeting drugs, including taxol. However, the role of HDAC3 in resistance to microtubule-targeting drugs in cancer cell lines in relation with tubulin β3 remains unknown. In this study, we investigated the molecular relationship between HDAC3 and tubulin β3. We showed that the low expression level of HDAC3 is related with the resistance to microtubule-targeting drugs. We showed that HDAC3 confers sensitivity to microtubule-targeting drugs. HDAC3 directly regulates the expression of MDR1 by binding to its promoter sequence. The expression of tubulin β3 was increased in cancer cell lines resistant to microtubule-

[1]Department of Biochemistry, College of Natural Sciences, Kangwon National University, Chunchon 200-701, Korea, [2]These authors contributed equally to this work.
*Correspondence: jeoungd@kangwon.ac.kr

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targeting drugs and HDAC3 decreased the expression of tubulin β3. HDAC3 showed direct binding to the promoter sequences of tubulin β3. HDAC6 showed an interaction with tubulin β3 that was disrupted by HDAC3. HADC6 acted upstream of tubulin β3, and the down-regulation of HDAC6 enhanced sensitivity to microtubule-targeting drugs. Tubulin β3 did not affect expression MDR1. The down-regulation of MDR1 did not affect the expression of tubulin β3, either. Our results indicated that tubulin β3 is an independent target of HDAC3.

MATERIALS AND METHODS

Materials

Anti mouse and anti rabbit IgG-horse radish peroxidase conjugate antibodies were purchased from Pierce Company. An ECL (enhanced chemiluminiscence) kit was purchased from Amersham. Lipofectamin and Plus™ reagent were purchased from Invitrogen (USA). Bioneer (Korea) synthesized all primers and oligonucleotides oused in this study. All antibodies used in this study were purchased from Santa Cruz Company.

Cell lines and cell culture

Cancer cell lines used in this study were cultured in Dulbecco's modified minimal essential medium (DMEM; Gibco, USA) supplemented with heat-inactivated 10% fetal bovine serum (FBS, Gibco) and antibiotics at 37°C in a humidified incubator with a mixture of 95% air and 5% CO_2. Cancer cell lines ($SNU387^R$ and $Malme3M^R$) made resistant to microtubule-targeting drugs were established by stepwise addition of the respective drug. Cells surviving drug treatment (attached fraction) were obtained and used throughout this study. SNU387/$SNU387^R$ or Malme3M/$Malme3M^R$ cells that stably express anti-sense HDAC3 cDNA, HDAC3-Flag or mutant HDAC3 were selected by G418 (400 μg/ml).

Western blot analysis

Western blot analysis, immunoprecipitation and cellular fractionation were performed according to the standard procedures (Kim et al., 2010b).

Cell viability determination

The cells were assayed for their growth activity using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma). Viable cell number counting was carried out by trypan blue exclusion assays.

HDAC3 constructs

$HDAC3^{S424A}$-Myc/$His_{(6)}$ expression plasmid (catalytically inactive HDAC3 mutant) was derived from pHDAC3-Myc/$His_{(6)}$ with the Quick-change site-directed mutagenesis kit (Stratagene).

Transfection

All transfections were performed according to the manufacturer's instructions. Lipofectamine and Plus reagents (Invitrogen) were used.

Histone deacetylase activity assays

Histone deacetylase activity was measured according to the manufacturer's instructions (Cayman Chemical, USA). The activity was measured according to the manufacturer's instructions. For immunoprecipitation, cells were lysed with ice-cold buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 15 mM MgCl2, 250 mMsucrose, 0.12 mM EDTA, 0.5% Nonidet P-40, and a mixture of protease inhibitors). The lysates were suspended with nuclear extraction buffer (50 mM HEPES, pH 7.5, 420 mM NaCl, 0.5 mMn EDTA, 0.1 mM EGTA, and 10% glycerol), sonicated for 30 s, and centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant containing the nuclear extract was immunoprecipitated with anti-HDAC3 (2 μg/ml), anti-HDAC2 (2 μg/ml), or anti-IgG antibody (2 μg/ml). The immunoprecipitants were incubated with 200 μM acetylated fluorometric substrate for 30 min at 37°C, and 40 μl of developer was added. After 15 min, the fluorescencewas measured using an excitation wavelength of 340-360 nm and an emission wavelength of 440-460 nm.

Caspase-3 activity assays

Caspase-3 activity was measuredaccording to the manufacturer's instructions (BioVision, USA). Cells were lysed in 0.1 M HEPES buffer, pH 7.4, containing 2 mM dithiothreitol, 0.1% CHAPS, and 1% sucrose. Cell lysates were incubated with a colorimetric substrate, 200 μM Ac-DEVD-*p*-nitroanilide, for 30 min at 30°C. The fluorescence was measured at 405 nm using a microtiter plate reader.

ChIP assays

Assays were performed according to manufacturer's instruction (Upstate). The immunoprecipitates were reverse cross-linked. PCR was done on the phenol-chloroform-extracted DNA. PCR was done on the phenol-chloroform-extracted DNA with specific primers of tubulin β3 promoter-1 [5′-GCAGCAGTCGCCCAAGCAGA-3′ (sense)] and [5′-CAGCCCACCTGCACTGAGCC-3′ (antisense)], tubulin β3 promoter-2 [5′-GCTCAGTGCAGGTGGGCTGG-3′ (sense)] and [5′-CCTGCCCCACAGTGTGCTCG-3′ (antisense)], HDAC6 promoter-1[5′-TACAGAAACACCTGTGACCC-3′ (sense)] and [5′-ATCTGTGCTGTAGTGTCAGG-3′ (antisense)], HDAC6 promoter-2 [5′-TGCTTATCTCTCCGGTCCCA-3′ (sense)] and [5′-CTGCGGTGCAAGCTTTTTCT-3′ (antisense)], HDAC6 promoter-3 [5′-AGAAAAAGCTTGCACCGCAG-3′ (sense)] and [5′-CCCCATTCCCAGACCCTCTA-3′ (antisense)] sequences were used.

Preparation of SiRNA duplexes

The SiRNA duplexes were constructed with the following target sequences. Tubulin β3, sense (5′-AAGCCTCTTCCTCACAAGTACGCCTGTCTC-3′); antisense (5′-AACGGAGAAGAGTGTTCATGCCCTGTCTC-3′); HDAC6, sense (5′-AAGGTGTCACCTGAGGGTTATCCTGTCTC-3′); antisense (5′-AAATAACCCTCAGGTGACACCCCTGTCTC-3′); MDR1-1, sense (5′-AATCCAAGGCATCAATTTCACCCTGTCTC-3′); antisense (5′-AAGTGAAATTGATGCCTTGGACCTGTCTC-3′); MDR1-2, sense (5′-AATTGCATACGCTAAGAGTTCCCTGTCTC-3′); antisense (5′-AAGAACTCTTAGCGTATGCAACCTGTCTC-3′); Control, sense (5′-AATTCTCCGAACGTGTCACGTCCTGTCTC-3′); antisense (5′-AAACGTGACACGTTCGGAGAACCTGTCTC-3′). The construction of SiRNA was carried out according to the instruction manual provided by the manufacturer (Ambion, USA).

Statistical analysis

Statistical differences in this were determined by using the Student's *t* test.

RESULTS

Taxol-resistant cancer cell lines show lower expression level of HDAC3 than taxol-sensitive cancer cell lines

We established cancer cell lines selected for resistance to

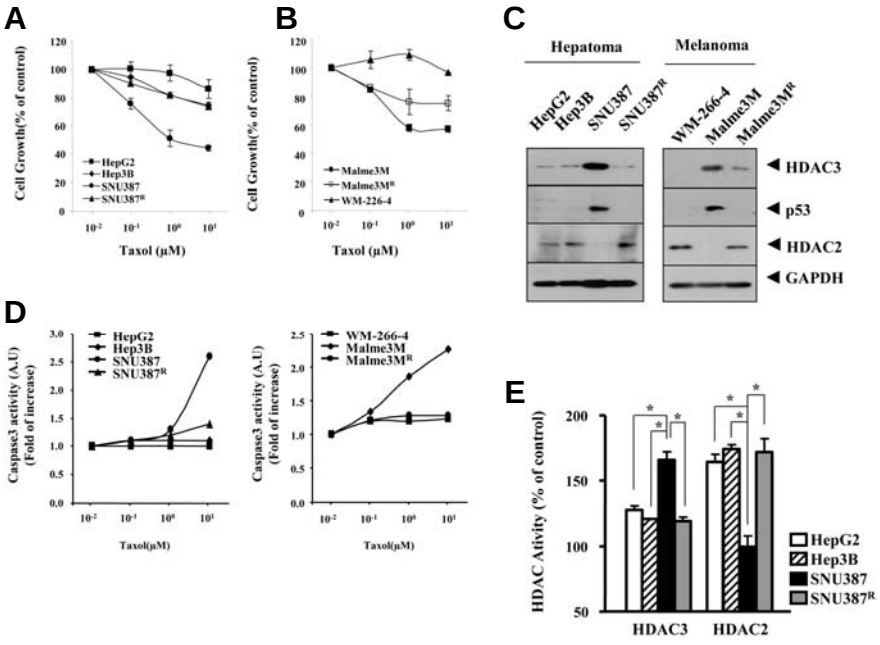

Fig. 1. Low expression of HDAC3 is correlated with resistance to taxol. (A) The indicated hepatoma cell line was treated with various concentrations of taxol for 48 h, followed by MTT assays. Hep3B, SNU387 and SNU387^R are the hepatocellular carcinoma cell lines, and HepG2 is a hepatoma cell line. The numbers are average of three independent experiments. Each experiment consists of triplicate measurement. (B) The indicated hepatoma cell line was treated with various concentrations of taxol for 48 h, followed by MTT assays. Each value represents an average obtained from 3 independent experiments. Data is expressed as a mean ± SD. (C) Cell lysates from the indicated cancer cell line were subjected to Western blot analysis. The representative figures are provided from three independent experiments. (D) The indicated hepatoma cell line was treated with various concentrations of taxol for 48 h, followed by MTT assays. (E) Cell lysates isolated from the indicated cancer cell line were subjected to HDAC activity assays as described. *p < 0.05.

celastrol, a microtubule-targeting drug. We examined the role of HDAC(s) in resistance to microtubule-targeting drugs such as celastrol and taxol. Hepatoma cell lines i.e., HepG2, Hep3B and SUN387^R cells showed resistance to taxol (Fig. 1A). Melanoma cell line WM266-4 and Malme3MR showed resistance to taxol (Fig. 1B). SNU387^R and Malme3MR cells are cancer cells selected for resistance to celastrol. HepG2, Hep3B and SUN387^R showed lower expression of HDAC3 than taxol-sensitive SNU387 cells (Fig. 1C). Furthermore, WM266-4 and Malme3M^R showed lower expression of HDAC3 than taxol-sensitive Malme3^M cells (Fig. 1C). The expression of HDAC3 in these cancer cell lines showed correlation with the expression of p53 (Fig. 1C). HDAC3 expression was inversely related to HDAC2 expression (Fig. 1C). SNU387 and Malme3M cells showed higher caspase-3 activity than the respective controls (Fig. 1D). This finding suggested that sensitivity of SNU387 and Malme3M cells to taxol results from caspase-3 activation. HDAC3 expression level is correlated with HDAC3 activity, similar to HDAC2 (Fig. 1E). The expression level of HDAC3 and HDAC2 showed correlation with their activity in melanoma cells employed in this study (data not shown). These results suggested that the expression level of HDAC3 might determine the response to microtubule-targeting drugs.

HDAC3 confers sensitivity to microtubule-targeting drugs

Because the down-regulation of HDAC 3was correlated with resistance to taxol, we hypothesized that over-expression of HDAC3 confers sensitivity to microtubule-targeting drugs. In this study, we employed catalytically inactive mutant HDAC3^{S424A} to determine whether HDAC3 activity is necessary to confer sensitivity to microtubule-targeting drugs. Ser424 is a non-conserved residue among class I HDACs. Ser 424 is the the protein kinase CK2 phosphoacceptor site in HDAC3 (Zhang et al., 2005). HDAC3^{Ser424} lacks histone deacetylase activity (Zhang et al., 2005). Wild type HDAC3, but not HDAC3^{S424A}, enhanced the sensitivity of HepG2, Hep3B and WM-266-4 cells (Figs. 2A, 2C, and 2E) to microtubule-targeting drugs such as celsatrol, taxol and vinblastine. The enhanced sensitivity was accompanied by enhanced cleavages of PARP and caspase-3 in these cancer cells (Figs. 2B, 2D, and 2F). These results suggested that HDAC3 activity is necessary for conferring sensitivity to microtubule-targeting drugs. We established cancer cell lines that stably express anti-sense HDAC3 (SNU387-As-HDAC3, Mame3M-As-HDAC3) to further confirm the role of HDAC3. SNU387-As-HDAC3 cells and Malme3M-As-HDAC3 cells showed higher resistance to microtubule-targeting drugs than the respective controls (Table 1). They also showed increased expression of MDR1 (Figs. 3A and 3B). Wild type HDAC3, but not HDAC3^{Ser424A}, enhanced cleavages of PARP and caspase-3 in SNU387-As-HDAC3 and Malme3M-As-HDAC3 in response to microtubule-targeting drugs (Figs. 3C and 3D). Wild type HDAC3, but not HDAC3^{S424A}, enhanced the sensitivity of SNU387-As-HDAC3 cells and Malme3M-As-HDAC3 cells to microtubule-targeting drugs (Table 1). This result confirmed that resistance to microtubule-targeting drugs results from the down-regulation of HDAC3. Taken together, these results suggested that HDAC3 regulates response to microtubule-targeting drugs.

HDAC3 directly regulates the expression of tubulin β3

Over-expression of tubulin β3 is involved in resistance to microtubule-targeting drugs (Kavallaris et al., 1997). We hypothesized that HDAC3 would be a negative regulator of tubulin β3. SNU387^R and Malme3M^R cells showed higher expression levels of tubulin β3 than SNU387 and Malme3M cells (Fig. 4A). HDAC6 interacts with tubulin β3 and causes deacetylation (Zhang et al., 2003). HDAC6 showed an interaction with tubulin β3 in SNU387^R and Malme3M^R cells (Fig. 4A). SNU387-AS-HDAC3 and Malme3M-AS-HDAC3 cells stably expressing anti sense HDAC3 showed increased expression of HDAC6 and tubulin β3 (Fig. 4B), suggesting that HDAC3 may act as a

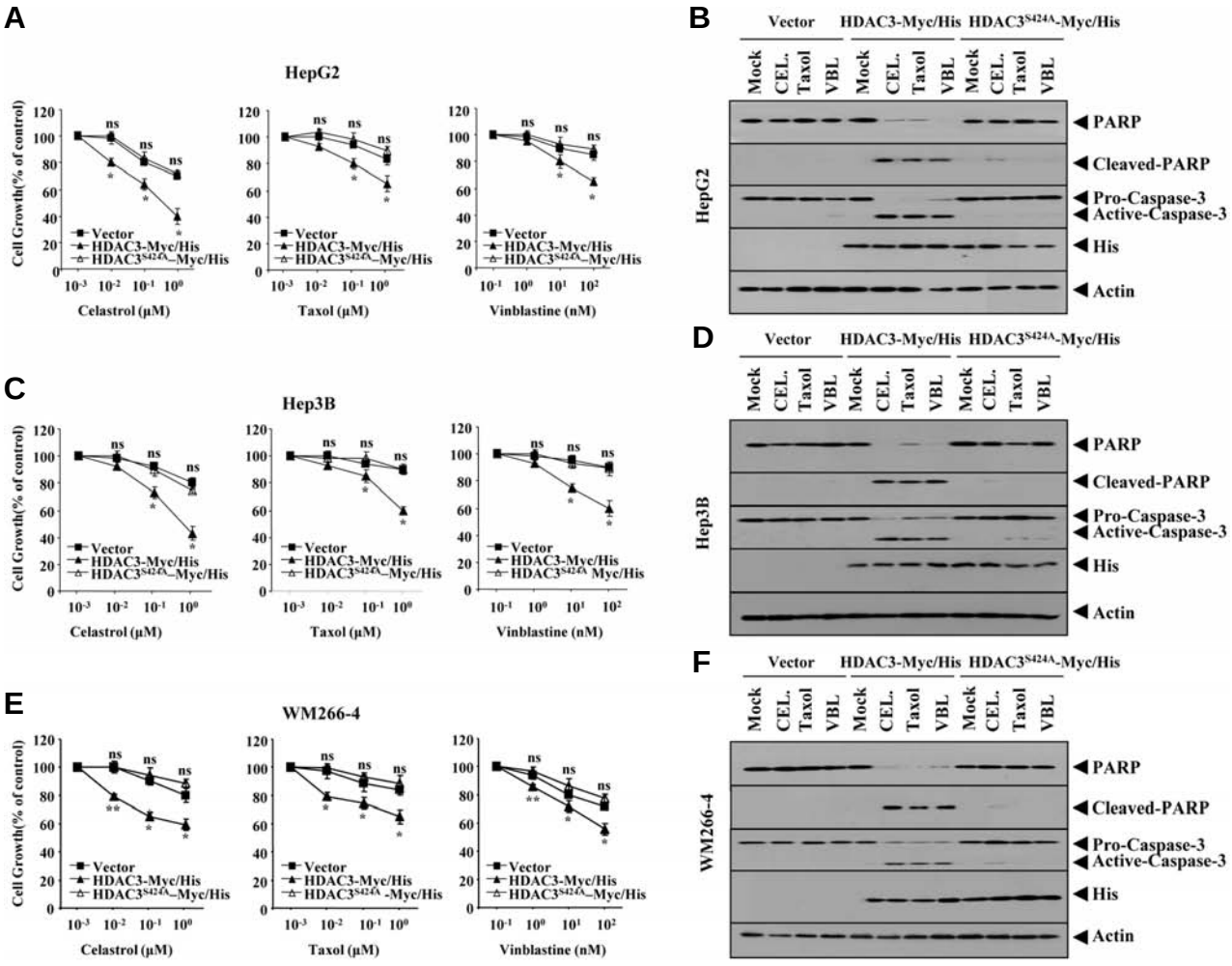

Fig. 2. HDAC3 confers sensitivity to celastrol, taxol and vinblastine in cancer cell lines that express low levels of HDAC3. Each indicated drug-resistant cancer cell line was transiently transfected with control vector (1 μg), HDAC3-His (1 μg) or HDAC3 (S424A)-His (1 μg). The next day, cells were treated with or without various concentrations of the indicated drugs for 24 h. MTT assays were performed (A, C, and E). *$p < 0.005$; **$p < 0.0005$. P value was determined in comparison with value obtained from HepG2, Hep3B or WM-266-4 cells transfected with control vector. NS denotes not significant. Each indicated drug-resistant cancer cell line was transiently transfected with control vector, HDAC3-Myc/His or HDAC3^{S424A}-Myc/His. The next day, cells were treated with the indicated drugs (1 μM for celastrol and taxol; 100 nM for vinblastine) for 24 h, followed by Western blot analysis (B, D, and F). VBL denotes vinblastine and CEL denotes celastrol.

negative regulator of HDAC6 and tubulin β3. Wild type HDAC3, but not mutant HDAC3 (S424A), decreased the expression of HDAC6 and tubulin β3 and prevented an interaction between HDAC6 and tubulin β3 (Fig. 4C). Celastrol and taxol led to the decreased expression of HDAC3 while increasing the expression of tubulin β3 (Fig. 4D), suggesting that HDAC3 and tubulin β3 are involved in anti-cancer drug-resistance. The decreased expression of HDAC3 preceded the increased expression of tubulin β3 by these anti cancer drugs (Fig. 4D), suggesting that HDAC3 functions upstream of tubulin β3. Tubulin β3 promoter contains putative binding sites for various transcription regulators such as DNMT1, Snail, HDAC2, AP1 and SP1 (Fig. 4E). ChIP assays showed the binding of HDAC3 to the promoter sequences of tubulin β3 (Fig. 4F). The down-regulation of tubulin β3 increased sensitivity to microtubule-targeting drugs via apoptosis (Figs. 5A and 5B). The down-regulation of HDAC6 decreased the expression of tubulin β3 and MDR1, but not HDAC3 in SNU387^R and Malme3M^R cells (Fig. 5C). The down-regulation of tubulin β3 did not affect the expression of HDAC6 or MDR1 (Fig. 5C). Taken together, these results suggested that tubulin β3 serves as an independent target of HDAC3 and HDAC6.

HDAC3 shows binding to the promoter sequences of HDAC6

Because HDAC3 exerted a negative regulation on the expression of HDAC6 (Fig. 4B), we examined the possibility of direct regulation of HDAC6 by HDAC3. HDAC6 promoter contains putative binding sites for HDAC2, DNMT1, YY1, and Snail (Fig.6A). SNU387^R and Malme3M^R cells showed higher expression levels of HDAC1, -2 and -6 than their respective controls (Fig. 6B). SNU387^R and Malme3M^R cells also showed higher expression level of YY1 (Fig. 6B). YY1 interacts with histone acetyltransferases p300 and histone deacetylase 1 (HDAC1), HDAC2, and HDAC3 (Yao et al., 2001). The activity of YY1 is

Table 1. Drug-sensitivity and relative resistance of SNU387-As-HDAC3 and Malme3M-A-HDAC3 cell lines transiently transfected with wild type or mutant HDAC3. To determine IC_{50} values, SNU387 or Malme3M cells were treated with or without various concentrations of the indicated drugs for 48 h. To determine the effect of wild type or mutant HDAC3 on IC_{50} values, SNU387-As-HDAC3 or Malme3M-As-HDAC3 cells were transiently transfected with the indicated construct. At 24 h after transfection, cells were treated with or without the indicated drug at various concentrations for 48 h. MTT assays were performed.

	Drug IC_{50}[a] (RF)		
	Celastrol (μM)	Taxol (μM)	Vinblastine (nM)
SNU387	0.84 ± 0.039	0.82 ± 0.184	3.47 ± 0.102
SNU387-AS-HDAC3			
Vector	2.49 ± 0.020 (2.9[c])	2.75 ± 0.001 (3.3)	10.21 ± 0.021 (2.9)
HDAC3-His	1.08 ± 0.016 (1.2)	1.03 ± 0.030 (1.3)	4.27 ± 0.240 (1.2)
$HDAC3^{S424A}$-His	2.11 ± 0.031 (2.5)	2.38 ± 0.029 (3.0)	8.89 ± 0.207 (2.6)
Malme3M	0.60 ± 0.043[b]	0.50 ± 0.070	8.00 ±.230
Malme3M-AS-HDAC3			
Vector	1.89 ± 0.159 (3.1[c])	1.65 ± 0.205 (3.3)	17.04 ± 0.121 (2.1)
HDAC3-His	0.78 ± 0.019 (1.3)	0.63 ± 0.003 (1.2)	8.54 ± 0.020 (1.1)
$HDAC3^{S424A}$-His	1.75 ± 0.250 (2.9)	1.35 ± 0.107 (2.7)	16.09 ± 0.153 (2.0)

[a]IC_{50}, the concentration of drug required to inhibit cell growth by 50%. [b]Mean ± s.d. of at least 3 independent experiments. [c]RF, resistance factor (IC_{50} in SNU387-As-HDAC3 or Malme3M-As-HDAC3 transfected with control vector /IC_{50} in SNU387 or Malme3M cell line).

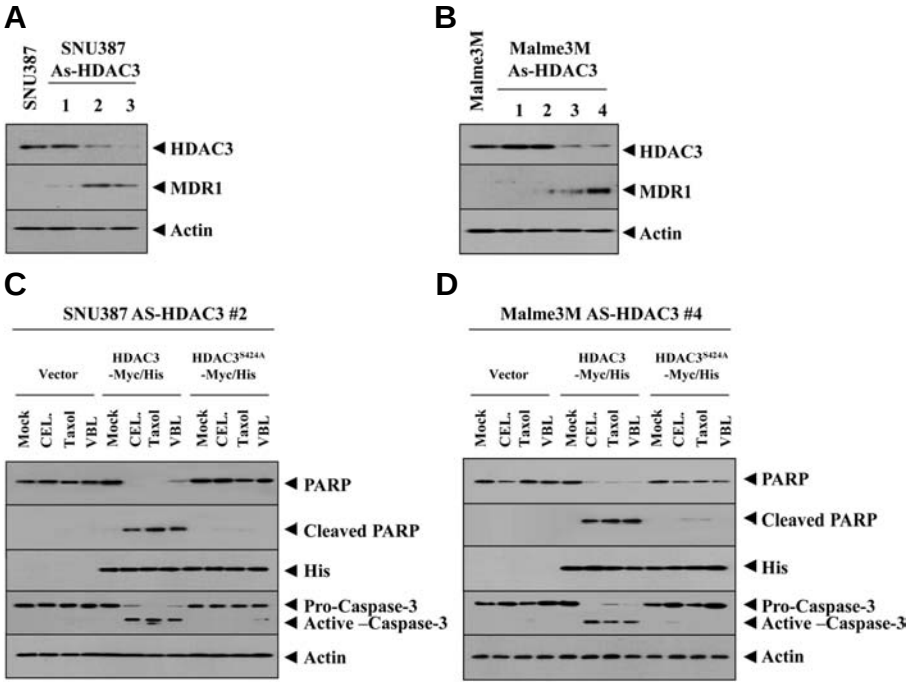

Fig. 3. Wild type, but not mutant HDAC3, confers sensitivity to microtubule-disrupting drugs in cancer cell lines that stably express antisense-HDAC3. Cell lysates from the indicated cell line were subjected to Western blot (A, B). (C) SNU387 cells that stably express antsense-HDAC3 (SNU387-As-HDAC3) were transiently transfected with control vector, HDAC3-Myc/His or $HDAC3^{S424A}$-Myc/His. At 24 h after transfection, cells were treated with or without celastrol (1 μM), taxol (1 μM) or vinblastine (100 nM) for 24 h, followed by Western blot analysis. (D) The same as C except that Malme3M-As-HDAC3 cell line was employed.

regulated through acetylation by p300 and deacetylation by HDACs. In other words, YYI is active in its deacetylated state. ChIP assay showed the binding of HDAC1 and -2 to the promoter sequences of HDAC6 (Fig. 6C). YY1 may recruit HDAC1 and/or HDAC2 to the promoter sequences of HDAC6. HDAC3 did not show binding to the site 1 of HDAC6 promoter (data not shown). We therefore focused on ChIP assays employing sites 2 and 3 of the HDAC6 promoter sequences. Wild type HDAC3, but not mutant HDAC3 (HDAC3 S424A), showed binding to HDAC6 promoter sequences in drug-sensitive Malme3M cells (Fig. 6C). This suggested the direct involvement of HDAC3 in the regulation of HDAC6 expression. HDAC3 exerted negative effects on the binding of HDAC1 and -2 to the promoter sequences of HDAC6 (Fig. 6C). It is probable that HDAC1 and -2 may be involved in resistance to microtubule-targeting drugs. $SNU387^{R}$ cells showed higher expression level of Ac-H3K9/14 and Ac-H4 K16 (data not shown). HDAC3 exerted a negative effect on the binding of Ac-H3K9/14 and Ac-H4 K16 to the promoter sequences of HDAC6 (Fig. 6C). In addition, YY1 showed binding to the promoter sequences of HDAC6 that was

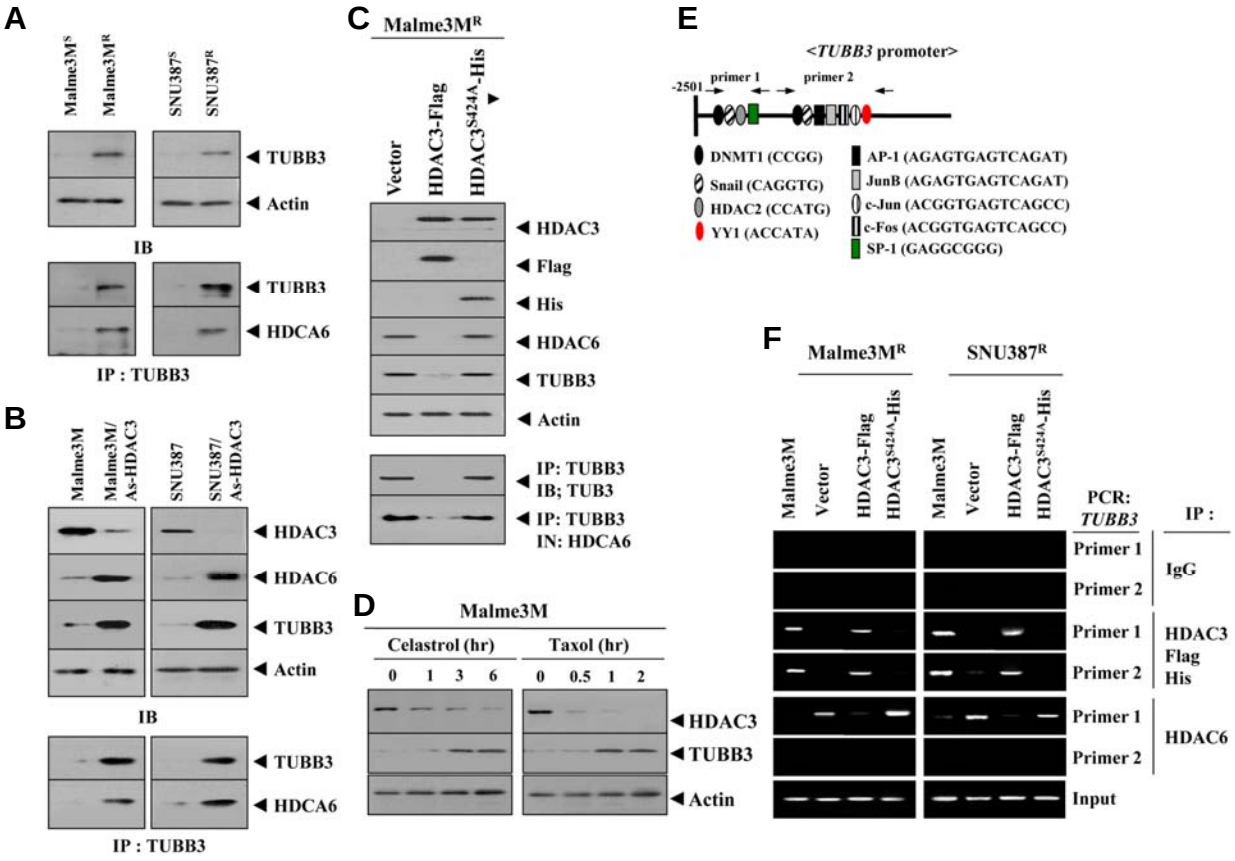

Fig. 4. HDAC3 regulates expression of tubulin β3 and the interaction between HDAC6 and tubulin β3. (A) Cell lysates from each cell line were immunoprecipitated with the indicated antibody (2 μg/ml), followed by Western blot (lower panel). Cell lysates were also subjected to Western blot (upper panel). (B) Cell lysates of the indicated cell line were immunoprecipitated with the indicated antibody (2 μg/ml), followed by Western blot (lower panel). Cell lysates were also subjected to Western blot (upper panel). (C) At 48 h after transfection with the indicated construct, cell lysates were immunoprecipitated with the indicated antibody (2 μg/ml), followed by Western blot (lower panel). Cell lysate were also subjected to Western blot (upper panel). (D) Malme3M cells were treated with celastrol (1 μM) or taxol (1 μM) for various time intervals. Cell lysates prepared at each time point were subjected to Western blot analysis. (E) Shows the proximal promoter sequences of tubulin β3. (F) At 48 h after transfection with the indicated construct, ChIP assays were performed. Cell lysates prepared from untransfected Malme3M or SNU387 cells were also subjected to ChIP assays.

prevented by HDAC3 (Fig. 6C). YY1 possibly interacts with HDAC1 and/or HDAC2, and HDAC3 may prevent this interaction by negatively regulating expression of HDAC1 and/or HDAC2. The down-regulation of HDAC6 did not affect the expression of HDAC1, HDAC3 or YY1 (Fig. 6D). This indicated that HDAC1, HDAC3 and YY1 function upstream of HDAC6. The down- regulation of HDAC6 decreased the expression of MDR1 and tubulin β3 (Fig. 6D). The down- regulation of HDAC6 enhanced sensitivity to taxol (Fig. 6E). The down-regulation of HDAC6 enhanced sensitivity to celastrol (data not shown). Taken together, these results showed the regulatory role of HDAC3 in the expression of HDAC6. These results also showed that HDAC6 is a downstream target of HDAC3 and functions upstream of tubulin β3 and MDR1.

MDR1 is necessary for resistance to microtubule-targeting drugs

Because HDAC3 regulated expression of MDR1 (Figs. 3A and 3B), we examined the role of MDR1 in resistance to microtubule-targeting drugs and the relationship between MDR1 and tubulin β3. The down-regulation of MDR1 enhanced sensitivity to microtubule-targeting drugs (Fig. 7A). The down- regulation of MDR1 enhanced the microtubule-targeting drugs induced cleavages of PARP (Fig. 7B). Over- expression of HDAC3 in Malme3M^R or SNU387^R cells in the presence of MDR1 knock down did not further enhance sensitivity to microtubule-targeting drugs (Fig. 7C) or affect the knock down state of MDR1 (Fig. 7D). This suggested that MDR1 serves as a downstream target of HDAC3. HDAC3 decreased the expression of tubulin β3 (Fig. 7D). Tubulin β3 mediates resistance to microtubule-targeting drugs (Cittelly et al., 2012). The down-regulation of MDR1 did not affect expression of tubulin β3 (Fig. 7D), suggesting that MDR1 and tubulin β3 may serve as independent targets of HDAC3. Taken together, these results suggested that MDR1 serves as a downstream target of HDAC3 that affects response to microtubule-targeting drugs.

DISCUSSION

Microtubule-destabilizer-resistant cancer cell lines show an

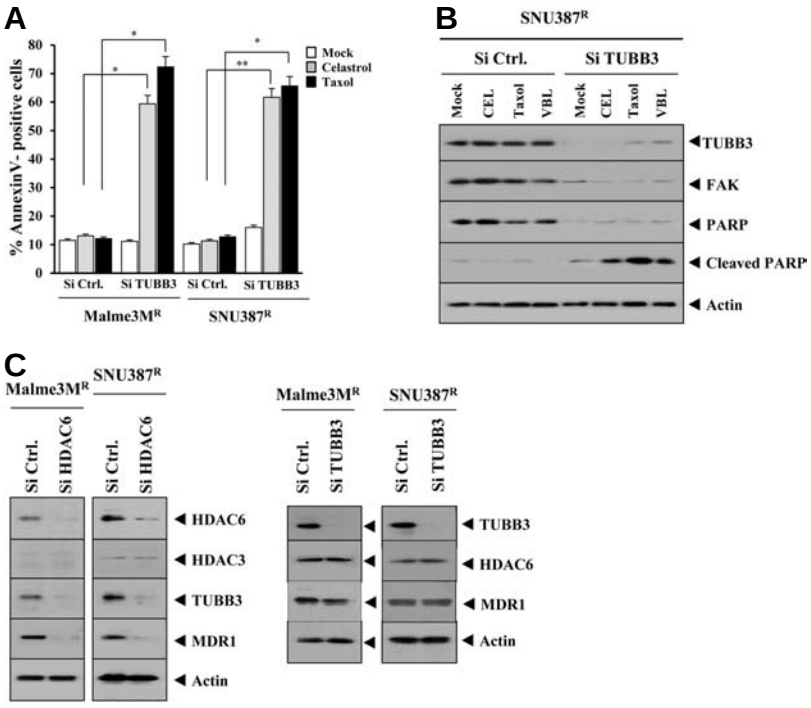

Fig. 5. Tubulin β3 acts downstream of HDAC6, and the down-regulation of tubulin β3 enhances sensitivity to microtubule-targeting drugs. (A) The indicated cell line was transfected with the indicated siRNA (10 nM each). The next day, cells were treated with various concentrations of the indicated drug for 24 h, followed by annexin V-FITC staining. *$p < 0.05$; **$p < 0.005$. A comparison was made between SNU387^R or Malme3M^R cells transfected with control siRNA. (B) The same as (A) except that Western blot was performed. (C) At 48 h after transfection with the indicated siRNA (10 nM each), Western blot was performed.

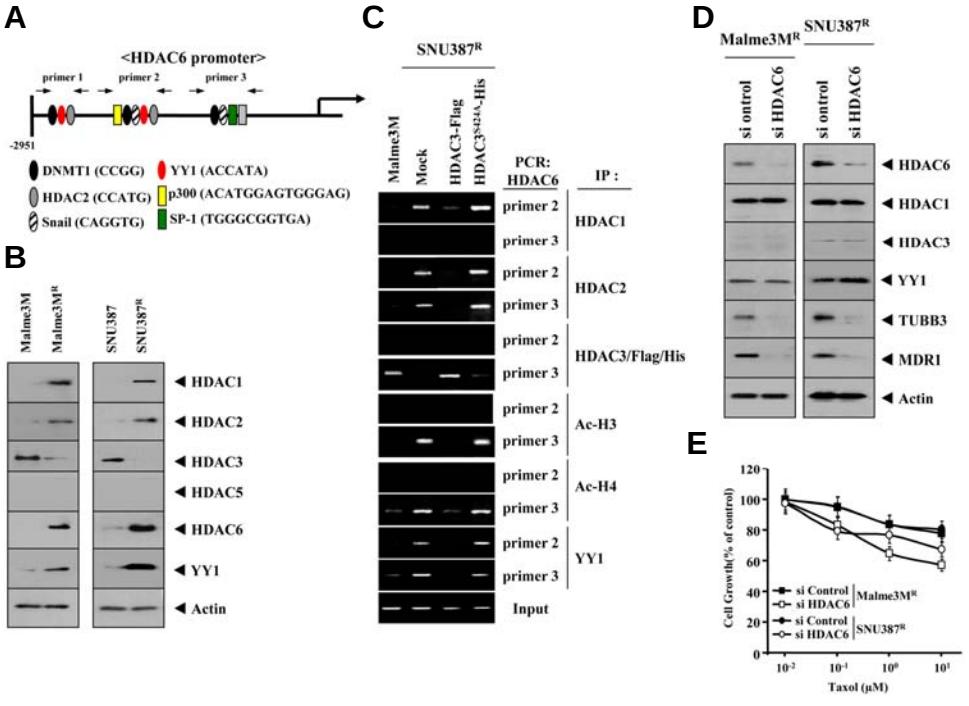

Fig. 6. HDAC3 directly regulates the expression of HDAC6 and the down-regulation of HDAC6 enhances sensitivity to taxol. (A) Shows the promoter sequences of the HDAC6. (B) Cell lysates isolated from the indicated cell line were subjected to Western blot analysis. (C) Cell lysates of the indicated cell line were immunoprecipitated with the indicated antibody (2 μg/ml), followed by ChIP assays. (D) SNU387^R or Malme3M^R cells were transfected with the indicated siRNA (10 nM each). At 48 h after transfection, cell lysates were prepared and subjected to Western blot analysis. (E) SNU387^R or Malme3M^R cells were transfected with the indicated siRNA (10 nM each). At 24 h after transfection, cells were treated with various concentrations of taxol for 24 h, followed by MTT assays.

increased expression of survivin (Hei et al., 2010). Therefore SNU387^R and Malme3M^R cells may show the increased expression of anti-apoptotic proteins such as survivin. The down-regulation of anti-apoptotic prohibin enhances sensitivity to taxol (Patel et al., 2010). The enhanced sensitivity to microtubule-targeting drugs is related with caspase-3–depedent pathway as evidenced by cleavage of PARP (Figs. 3C and 3D). It will therefore be necessary to examine the effect of HDAC3 on caspase-independent cell death by examining the expression of anti-apoptotic proteins such as surviving and prohibin.

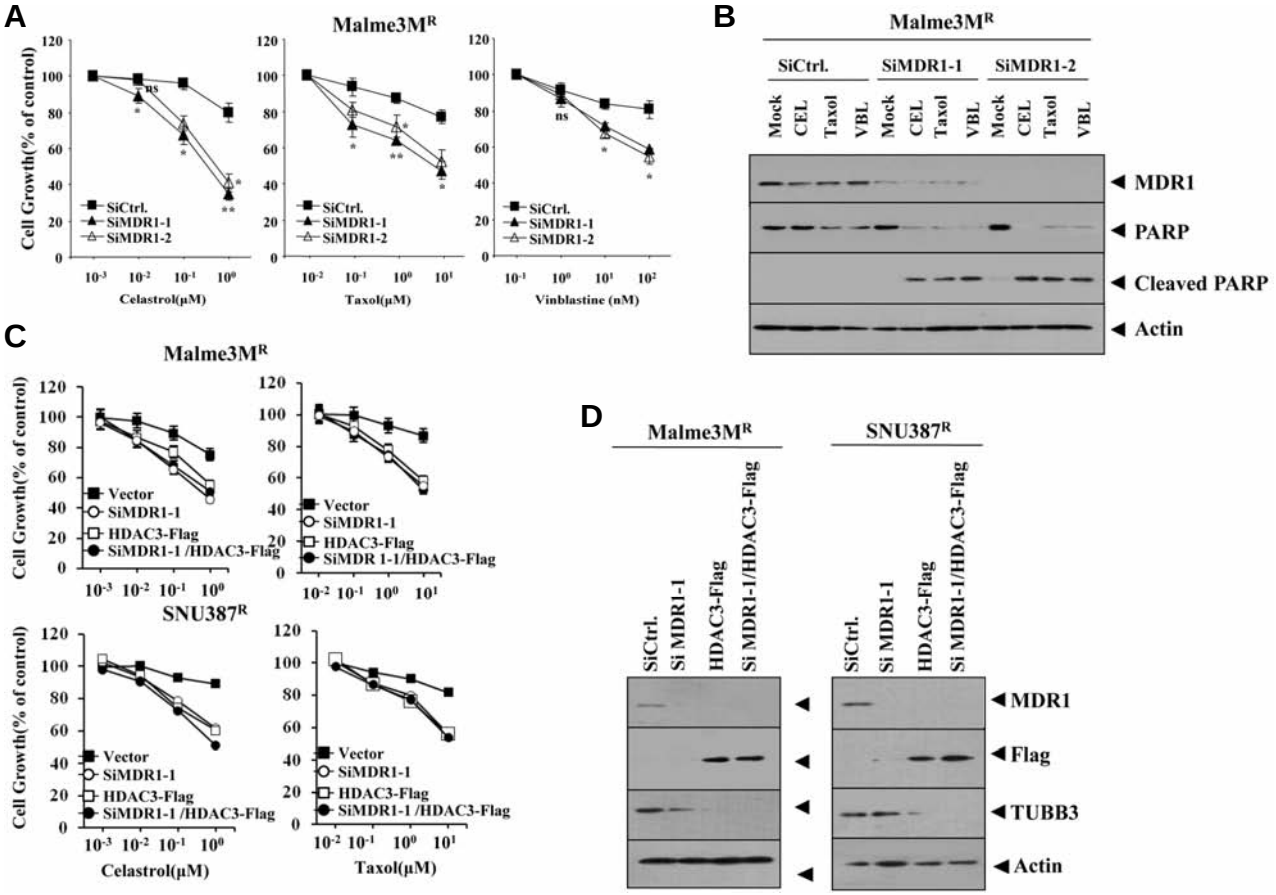

Fig. 7. MDR1 is necessary for resistance to microtubule-disrupting drugs. (A) Malme3M^R cells were transfected with indicated siRNA. The next day, cells were treated with or without various concentrations of celastrol, vinblastine or taxol for 24 h. *p < 0.05; **p < 0.005. P value was determined in comparison with value obtained from Malme3M^R transfected with scrambled siRNA. (B) Malme3M^R cells were transfected with indicated siRNA (10 nM each). The next day, cells were treated with celastrol (1 μM), taxol (1 μM) or vinblastine (100 nM) for 24 h, followed by Western blot analysis. (C) The indicated cell line was transfected with the indicated construct alone or in combination. The next day, cells were treated with various concentrations of cealstrol or taxol for 24 h, followed by MTT assays. (D) The indicated cell line was transfected with the indicated construct alone or in combination. Western blot analysis was performed at 48 h after transfection.

The MDR1 expression level is correlated with resistance to taxol and doxorubicin (Mechetner et al., 1998). It will be interesting to examine whether HDAC3 would also confer sensitivity to doxorubicin and examine the direct regulation of MDR1 by HDAC3. It will be necessary to examine the direct binding of the HDAC3 to the promoter sequences of MDR1. CDX2, a transcription factor, regulates MDR1 expression (Takakura et al., 2010) and the down-regulation of CDX2 enhances sensitivity to cisplatin, doxorubicin and 5-FU (Yan et al., 2013). It will be necessary to examine the effect of HDAC3 on the expression of CDX2.

The down-regulation of HDAC6 leads to the degradation of EGFR (Gao et al., 2010). We found an increased expression of EGFR in Mame3M^R cells. In addition, Malme3M^R cells show resistance to EGFR inhibitors (data not shown). The inhibition of EGFR by cetuximab enhances sensitivity to taxol (data not shown). It is probable that EGFR signaling may be responsible for the decreased expression of HDAC3 in SNU387^R and Malme3M^R cells. It is also reasonable that HDAC3 may exert a negative control on the expression and/or activity of EGFR in SNU837 and/or Malme3M cells.

Over-expression of tubulin β3 has been shown in paclitaxel-resistant cells (Kamath et al., 2005; Seve et al., 2010; Verdier-Pinard et al., 2003). We showed the over-expression of tubulin β3 in SNU387^R and Malme3M^R cells (Fig. 4A). It will be necessary to examine the role of signaling pathways such as MAPK and JNK on the expression of HDAC3 and tubulin β3. Tubulin β3 promoter contains putative binding sites for various transcription factors such as AP1, SP1, Snail and YY1 (Fig. 4E). For example, the expression of SP1 is increased in SNU387^R and Malme3M^R cells (data not shown). It is lijkely that the down-regulation of HDAC3 induces expression SP1, which in turn binds to the promoter sequences of tubulin β3 and induces the expression of tubulin β3 in SNU387^R and Malme3M^R cells.

Mutations in tubulin β1 confer resistance to taxol (Yin et al., 2010). It would be interesting to examine possible mutations in tubulin β1 and tubulin β1 expression level in SNU387^R and Malme3M^R cells. The selective inhibition of HDAC6 by Vorinostat leads to the enhanced sensitivity to taxol (Owonikoko et al., 2010). Furthermore, the down-regulation of HDAC6 enhanced sensitivi-

ty to taxol (Fig. 6E). Depletion of HDAC6 enhances cisplatin-induced cytotoxicity by activating the ATR/Chk1 pathway (Wang et al., 2012). These reports indicated the role of HDAC6 in anti-cancer drug-resistance. Because HDAC3 regulates the expression of HDAC6, it will be interesting to examine the effect of HDAC3 on the ATR/Chk1 pathway. Tubacin, a selective inhibitor of HDAC6, enhances DNA damage induced by etoposide or SAHA as indicated by increased accumulation of γH2AX and activation of the checkpoint kinase Chk2 (Namdar et al., 2010). It will also be interesting to examine the effect of HDAC3 in response to DNA damaging drugs.

HDAC6 is necessary for angiogenesis via its interaction with and deacetylation of the actin-remodeling protein cortactin, in endothelial cells (Kaluza et al., 2011). Because HDAC3 regulates the expression of HDAC6, it would be interesting to examine the effect of HDAC3 on the acetylation of cortactin. It will be necessary to examine the effect of HDAC3 on the expression of various angiogenic factor(s). Because HDAC3 confers sensitivity to microtubule-targeting drugs, it possibly exerts a negative effect on tumor-induced angiogenesis. VEGF signaling induces anti cancer drug-resistance by upregulating MDR1 expression (Akiyama et al., 2012). Because HDAC3 negatively regulates MDR1 expression, HDAC3 likely regulates the expression of VEGF.

MicroRNAs (miRNAs) are non-coding RNA molecules that mediate posttranscriptional gene regulation and are strongly implicated in cellular processes such as cell proliferation, carcinogenesis, cell survival and apoptosis. MiRNA-binding factor Lin-28 mediates taxol-resistance in breast cancer cells (Lv et al., 2012). miR-148a attenuates taxol-resistance by regulating MSK1 expression (Fujita et al., 2010). miR-337-3p modulates taxol-sensitivity by targeting STAT3 and RAP1A (Du et al., 2012). These reports suggest the role of miRNAs in taxol-resistance. In this study, we identified miRNAs that were down-regulated in Malme3M^R cells. These include miR-138, -189, -211, - 324-3p and -335 (data not shown). It is necessary to further examine the effect of these miRNAs on HDAC3 expression and response to microtubule-targeting drugs. In conclusion, we showed a novel role of HDAC3 in determining response to microtubule-targeting drugs. We showed that HDAC3-HDAC6-tubulin β3 axis determines response to microtubule-targeting drugs.

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