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ORIGINAL ARTICLE

EZH2 supports nasopharyngeal carcinoma cell aggressiveness by forming a co-repressor complex with HDAC1/HDAC2 and Snail to inhibit E-cadherin

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The enhancer of zeste homolog 2 (EZH2) is upregulated and has an oncogenic role in several types of human cancer. However, the abnormalities of *EZH2* and its underlying mechanisms in the pathogenesis of nasopharyngeal carcinoma (NPC) remain unknown. In this study, we found that high expression of EZH2 in NPC was associated closely with an aggressive and/or poor prognostic phenotype ($P<0.05$). In NPC cell lines, knockdown of EZH2 by short hairpin RNA was sufficient to inhibit cell invasiveness/metastasis both *in vitro* and *in vivo*, whereas ectopic overexpression of EZH2 supported NPC cell invasive capacity with a decreased expression of E-cadherin. In addition, ablation of endogenous Snail in NPC cells virtually totally prevented the repressive activity of EZH2 to E-cadherin, indicating that Snail might be a predominant mediator of EZH2 to suppress E-cadherin. Furthermore, co-immunoprecipitation (IP), chromatin IP and luciferase reporter assays demonstrated that in NPC cells, (1) EZH2 interacted with HDAC1/HDAC2 and Snail to form a repressive complex; (2) these components interact in a linear fashion, not in a triangular fashion, that is, HDAC1 or HDAC2 bridge the interaction between EZH2 and Snail; and (3) the EZH2/HDAC1/2/Snail complex could closely bind to the *E-cadherin* promoter by Snail, but not YY1, to repress E-cadherin. The data provided in this report suggest a critical role of EZH2 in the control of cell invasion and/or metastasis by forming a co-repressor complex with HDAC1/HDAC2/Snail to repress E-cadherin, an activity that might be responsible, at least in part, for the development and/or progression of human NPCs.
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Introduction

Nasopharyngeal carcinoma (NPC) is a remarkably distinctive type of head and neck cancer, which is highly prevalent in Southern China and Southeast Asia. The incidence of NPC in Southern Chinese is about 25–30 per 100 000 persons per year (Lo *et al*., 2004). NPC has a high potential to metastasize to cervical lymph nodes and/or distant organs, and prevention of this metastasis is important for a favorable outcome (Fandi *et al*., 1994; Wei and Mok, 2007). Thus, it is necessary to elucidate the molecular mechanism(s) underlying tumorigenesis and metastasis of NPC, so as to find novel therapeutic targets and develop new modalities of treatment.

Enhancer of zeste homolog 2 (EZH2), a central member of polycomb repressive complexes (PRCs), which has intrinsic histone methyltransferase activity, has been found to contribute to the maintenance of cell identity, cell cycle regulation and oncogenesis (Varambally *et al*., 2002; Simon and Lange, 2008). Recently, there is increasing evidence that overexpression of the *EZH2* gene occurs in a variety of human malignancies, including oral, esophageal, gastric, colon, hepatocellular, bladder, prostate, breast, ovarian and endometrial cancers (Mimori *et al*., 2005; Raman *et al*., 2005; Sudo *et al*., 2005; Bachmann *et al*., 2006; Collett *et al*., 2006; Matsukawa *et al*., 2006; Kidani *et al*., 2009; He *et al*., 2010; Rao *et al*., 2010). Abnormalities of this gene were observed to correlate closely with tumor aggressiveness and/or poor patient prognosis. In our recent studies, we observed that knocking down EZH2 expression in hepatocellular carcinoma cells was sufficient to significantly reverse tumorigenicity in an athymic nude mouse model (Chen *et al*., 2007), and found that EZH2 protein

may serve as a promising diagnostic biomarker for hepatocellular carcinomas (Cai *et al*., 2011). To date, however, the molecular status of *EZH2* and its potential oncogenic role and molecular mechanisms in NPC has not been clearly elucidated. In this study, to investigate whether or not abnormalities of *EZH2* is involved in the pathogenesis of NPC, the protein expression dynamics and amplification status of *EZH2* were first examined in a series of carcinomarous and non-neoplastic human nasopharyngeal tissues and cells. In addition, the tumorigenicity of *EZH2* and the underlying molecular mechanisms involving the oncogenic role of *EZH2* were investigated.

Results

Expression of EZH2 in NPC cells and nasopharyngeal tissues and its correlation with NPC patients clinicopathological features and survival

Of the six NPC cell lines analyzed by western blot, five lines (that is, CNE1, CNE2, HNE1, 5-8F and C666) had higher levels of endogenous EZH2 expression than that in non-neoplastic nasopharyngeal control tissues, whereas the 6-10B cell line had low-level expression of EZH2 (Figure 1a, left). In primary NPCs, 9/13 (69%) had upregulated EZH2 expression, when compared with adjacent non-neoplastic nasopharyngeal tissues.

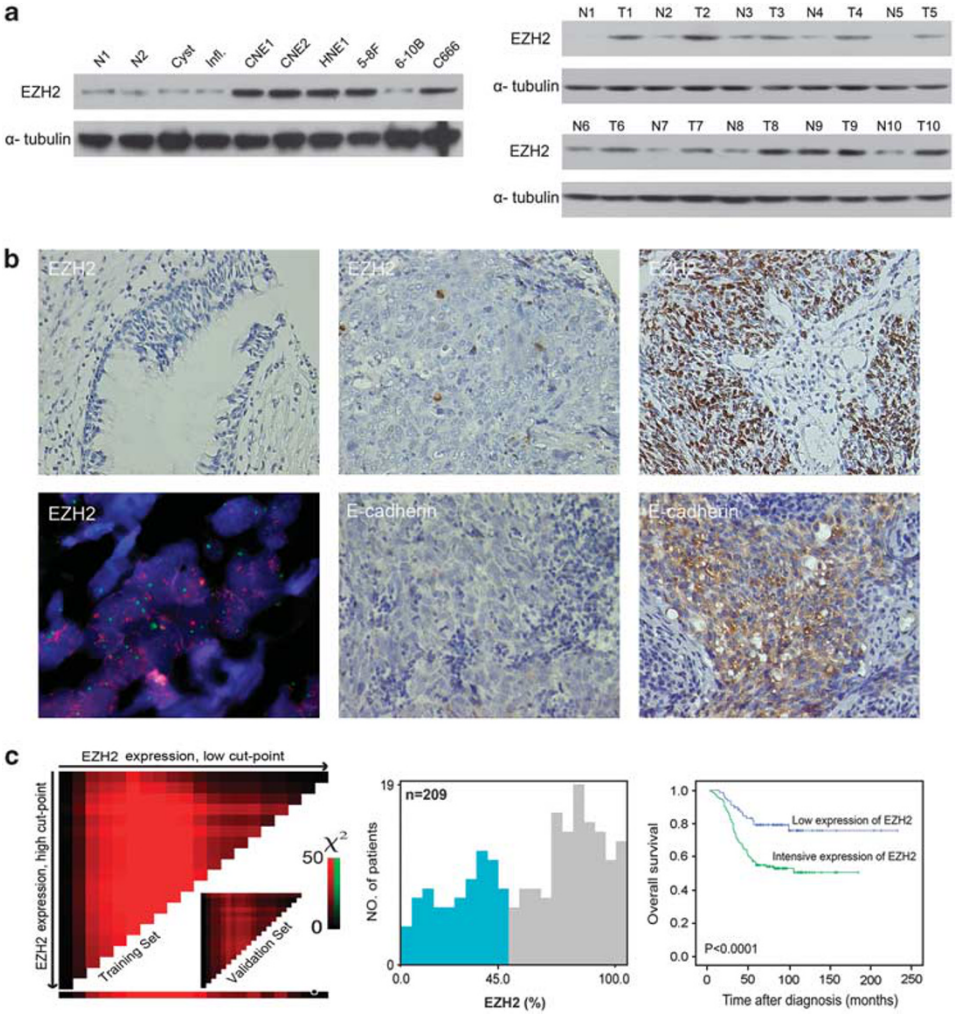

Figure 1 Expression and amplification of *EZH2* in NPC cells and nasopharyngeal tissues and prognostic significance in NPC patients. (**a**) The levels of EZH2 protein in six NPC cell lines and nasopharyngeal benign tissues examined by western blot (left); western blot showed that EZH2 expression was upregulated in 9 of the 10 NPC tissues compared with paired normal tissues (right), *N*, normal tissue; *T*, carcinoma tissue; *Infl*., inflammation tissue. (**b**) Upper panels, representative images showed negative expression of EZH2 by IHC in normal nasopharyngeal mucosa (left), low expression of EZH2 in NPC case 32 (middle) and high expression of EZH2 in NPC case 68 (right). Lower panels, amplification of *EZH2* gene was examined by fluorescence *in situ* hybridization in NPC case 68 (left); representative cases demonstrated negative expression of E-cadherin in NPC case 43, and positive expression of E-cadherin in the NPC case 76 (right). (**c**) X-tile plots of the prognostic marker EZH2. X-tile analysis was carried out on patient data from the NPC cohort, equally divided into training and validation subsets. X-tile plots of training sets were displayed in the left panels, with matched validation sets in the smaller inset. The plot shows the χ^2 log-rank values. The cut-point highlighted by the black/white circle in the left panels, is shown on a histogram of the entire cohort (middle panels), and a Kaplan–Meier plot (right panels). *P*-values were defined by using the cut-point derived from a training subset to produce a separate validation subset.

The nine NPC cases with upregulated expression of EZH2 are shown in Figure 1a, right.

Using immunohistochemistry (IHC) staining, high expression of EZH2 was observed in 131/209 (62.7%) of primary NPC tissues compared with only 3/50 (6%) of non-neoplastic nasopharyngeal mucosa (Figure 1b). Correlation analysis demonstrated that high expression of EZH2 was positively associated with a more advanced clinical stage of NPC ($P<0.01$, Table 1). Kaplan–Meier analysis showed that the mean disease-free survival time for patients with NPCs having a high expression of EZH2 was 112.6 months compared with 187.3 months for patients with NPCs having a low expression of EZH2 ($P<0.0001$, log-rank test, Figure 1c, Table 2). Further multivariate Cox regression analysis evaluated that EZH2 expression is an independent prognostic factor for poor survival of NPC patients (relative risk: 2.002, confidence interval: 1.151–3.482, $P=0.014$, Table 2).

In our study, amplification of *EZH2* gene was further examined by fluorescence *in situ* hybridization in nasopharyngeal tissue. Fluorescence *in situ* hybridization analysis was informative in 135/209 NPCs. Results showed that amplification of *EZH2* (Figure 1b) was evident in 11/135 (8.1%) of the informative NPCs.

Table 1 Correlation between clinico-pathological features and expression of EZH2 in 209 cases of human NPC

	EZH2 protein			P-*value*[a]
	All cases	*Low expression*	*High expression*	
Sex				0.337
Female	59	19 (32.2%)	40 (67.8%)	
Male	150	59 (39.3%)	91 (60.7%)	
Age at diagnosis (years)				0.820
⩽45	89	34 (38.2%)	55 (61.8%)	
>45	120	44 (36.7%)	76 (63.3%)	
Histological classification (WHO)				0.960
Type II	54	20 (37.0%)	34 (63.0%)	
Type III	155	58 (37.4%)	97 (62.6%)	
T status				0.005
T1	26	12 (46.2%)	14 (53.8%)	
T2	68	27 (39.7%)	41 (60.3%)	
T3	69	32 (46.4%)	37 (53.6%)	
T4	46	7 (15.2%)	39 (84.8%)	
N status				0.000
N0	40	22 (55.0%)	18 (45.0%)	
N1	96	44 (45.8%)	52 (54.2%)	
N2	51	9 (17.6%)	42 (82.4%)	
N3	22	3 (13.6%)	19 (86.4%)	
M status				0.003
M0	158	68 (43.0%)	59 (57.0%)	
M1	51	10 (19.6%)	41 (80.4%)	
Clinical stage				0.000
I	10	8 (80.0%)	2 (20.0%)	
II	55	27 (49.1%)	28 (50.9%)	
III	83	32 (38.6%)	51 (61.4%)	
IV	61	11 (18.0%)	50 (82.0%)	

Abbreviations: EZH2, enhancer of zeste homolog 2; NPC, nasopharyngeal carcinoma; WHO, World Health Organization.
[a]χ^2 test.

The expression levels of EZH2 influenced the invasive capacity of NPC cell lines in vitro

To investigate the impact of EZH2 on NPC cell line invasiveness, we first knocked down endogenous EZH2 in our CNE2 and HNE1 NPC cell lines using one of our previously constructed specific lenti-short hairpin RNAs (shRNAs) (Chen *et al*., 2007). Matrigel invasion assays demonstrated that ablation of endogenous EZH2 markedly reduced invasiveness of both CNE2 and HNE1 cell lines (Figure 2a). In addition, this reduction could be enhanced dramatically when the NPC cells were treated with Trichostatin A (TSA), a HDAC inhibitor (Figure 2b). These results indicate that the repression of EZH2 and inhibition of HDAC may synergistically inhibit NPC cell invasiveness. Next, we performed a series of reverse experiments to investigate whether elevated levels of EZH2 could restore the invasive ability of EZH2 repressed NPC cells. As anticipated, shEZH2-CNE2 and shEZH2-HNE1 cells regained high invasive capability when myc-EZH2, but not myc-EZH2ΔSET, were incorporated into NPC cells (Figure 2a).

To determine whether or not ectopic overexpression of EZH2 could enhance invasive capacity of NPC cells, we transfected myc-EZH2, myc-EZH2ΔSET or a control vector into our 6-10B NPC cell line (the cells with low levels of endogenous EZH2). The matrigel invasion assay showed that myc-EZH2/6-10B cells, but not myc-EZH2ΔSET/6-10B cells, had significantly increased invasive capacity, comparing with our control vector/6-10B cells. In addition, this ectopic EZH2-mediated invasive characteristic also could be sharply inhibited by incubating these cells with TSA (Figure 2a). Collectively, these results provide evidence that elevated expression levels of EZH2 are important for the aggressive phenotype of NPC cells, and in addition, the function of EZH2 in NPC cells invasiveness is dependent on its SET domain.

shRNA-mediated EZH2 knockdown reduced metastatic potential of NPC cell line in vivo

We evaluated the *in vivo* effects of EZH2 knockdown on NPC cell line invasion and/or metastasis using an experimental metastasis assay, in which lenti-shEZH2-CNE2 or control lenti-shLuc-CNE2 cells were injected into the lateral tail vein of 6-week-old athymic nude mice and the growth of these cells in the mouse lung was evaluated. The mouse lungs subsequently were examined under a dissection microscope. The control shRNA cells formed 6–37 metastatic nodules per lung in the 9 mice that were examined. In contrast, mice injected with EZH2 shRNA cells formed 0–5 nodules per lung ($n=9$ mice) (Figure 2c). Hematoxylin and eosin staining confirmed that both the number and the volume of micrometastatic lesions were markedly reduced in the

Table 2 Univariate and multivariate analysis of different prognostic parameters in 209 patients with NPC by Cox regression analysis

Variable	*Univariate analysis*			*Multivariate analysis*	
	All cases	*HR (95% CI)*	P-*value*	*HR (95% CI)*	P-*value*
Sex			0.547		
Female	59	1.0			
Male	150	0.863 (0.534–1.394)			
Age at surgery (years)			0.241		
⩽45	89	1.0			
>45	120	0.768 (0.494–1.194)			
Histological classification (WHO)			0.064		
Type II	54	1.0			
Type III	155	1.727 (0.969–3.078)			
T status			0.013		0.909
T1–T2	94	1.0		1.0	
T3–T4	115	1.800 (1.130–2.867)		0.982 (0.716–1.347)	
N status			0.000		0.962
N0–N1	136	1.0		1.0	
N2–N3	73	2.211 (1.421–3.440)		0.993 (0.733–1.344)	
M status			0.000		0.682
M0	158	1.0		1.0	
M1	51	2.783 (1.769–4.379)		0.858 (0.412–1.785)	
Clinical stage			0.000		0.007
I–II	65	1.0		1.0	
III–IV	144	3.106 (1.679–5.746)		2.520 (1.294–4.909)	
EZH2 expression			0.000		0.014
Low	78	1.0		1.0	
High	131	3.010 (1.715–5.284)		2.002 (1.151–3.482)	

Abbreviations: CI, confidence interval; EZH2, enhancer of zeste homolog 2; HR, hazards ratio; NPC, nasopharyngeal carcinoma; WHO, World Health Organization.

lungs of mice injected with lenti-shEZH2 (Figure 2d). These data indicate that EZH2 is necessary for the aggressive and highly metastatic phenotype of CNE2 NPC cells.

EZH2 negatively regulated E-cadherin expression in NPC cells

Since recent studies provide evidence that the suppression of EZH2 in gastric carcinoma cells resulted in an enhancement of the tumor-suppressor E-cadherin (Fujii and Ochiai, 2008), and that overexpression of EZH2 in a mammary epithelial cell line downregulates E-cadherin (Cao *et al*., 2008), we asked whether or not EZH2 regulates E-cadherin expression in our NPC cells. First, in our NPC–tissue microarray examined by IHC, we found a significant reverse correlation between expression of EZH2 and E-cadherin in our NPC cohorts ($P<0.001$, Fisher's exact test, Supplementary Table 1). Next, in NPC cell lines, we observed that lenti-shEZH2-mediated EZH2 knockdown did cause an increased expression of E-cadherin in both CNE2 and HNE1 cell lines. This upregulation of E-cadherin was accompanied by diminished trimethylation of H3K27 (H3K27me3), levels in both NPC cells (Figure 2b). In addition, the combination of EZH2 knockdown with treatment of TSA in the CNE2 or HNE1 cells resulted in a stronger upregulation of E-cadherin compared with EZH2 knockdown, although the combination treatment with TSA did not alter the total levels of H3K27me3 (Figure 2b). In reverse experiments, when we reintroduced myc-EZH2 in the EZH2 knocked down cells, the expression of E-cadherin was inhibited again while the levels of H3K27me3 increased. However, the reintroduction of myc-EZH2ΔSET did not have the same affect (Figure 2b). These data suggest that EZH2 negatively regulates E-cadherin expression, perhaps via trimethylation of H3K27, and possibly the SET domain of EZH2 is needed for EZH2-mediated E-cadherin regulation. In addition, it is possible that the HDAC inhibitor TSA could synergistically increase the protein levels of E-cadherin mediated by EZH2 depletion.

On the other hand, when ectopic overexpression of EZH2 in 6-10B NPC cells was observed, the levels of E-cadherin were reduced and accompanied by an increased expression of H3K27me3. Furthermore, the treatment of TSA partially restored the reduced levels of E-cadherin in myc-EZH2/6-10B cells, but the levels of H3K27me3 were not decreased (Figure 2b).

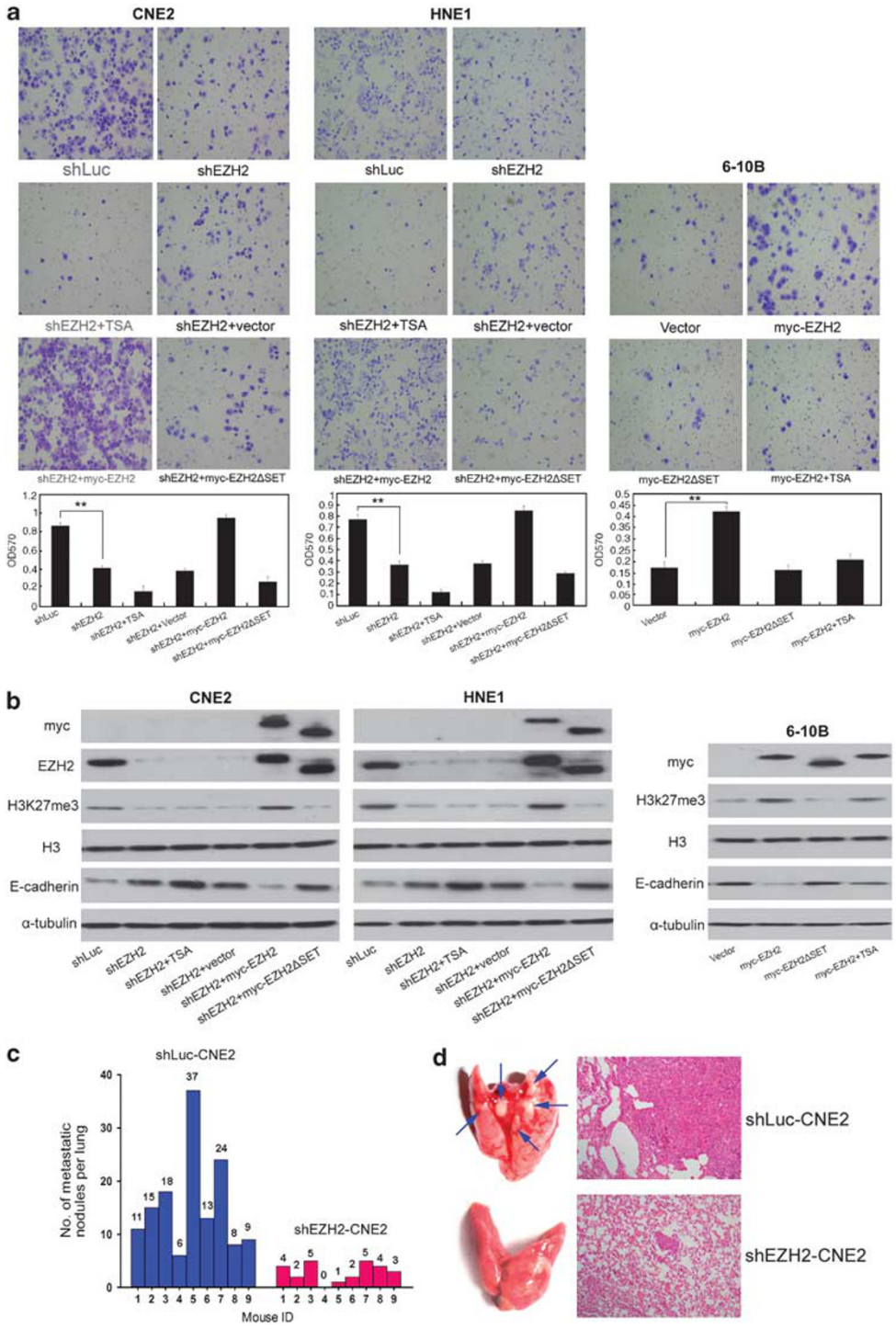

Figure 2 The role of EZH2 on invasiveness and metastasis of NPC cell lines. (**a**) Matrigel-coated Boyden chamber assay was performed to evaluate the invasive properties of CNE2, HNE1 and 6-10B cell lines, treatments indicated. Upper panel, representative areas of invaded cells stained with crystal violet. Lower panel, quantitation measured the absorbance at 570 nm using a spectrophotometer. *P*-values were calculated between control and EZH2 knockdown CNE2 and HNE1 cells, and between vector and ectopic EZH2 overexpressed 6-10B cells, using a Student's *t*-test. Data described are the means ± s.e. of three independent experiments. Error bars represent s.e. $^{**}P<0.01$. (**b**) Western blot analysis shows the levels of different proteins in CNE2, HNE1 and 6-10B cells, treatments indicated. The knockdown efficiency of EZH2, mediated by the lentivirus, was measured by antibodies against EZH2, and the expression levels of exogenous myc-EZH2 or myc-EZH2ΔSET was detected by antibodies against myc. The alteration of H3K27me3 and E-cadherin levels was examined by antibodies against H3K27me3 and E-cadherin, respectively. Histone H3 and α-tubulin were used as a loading control. Experiments were performed three times and a representative result is shown. (**c**) Number of metastases in lungs of mice ($n=9$ per group) 8 weeks after tail vein injection of control shLuc cells (average number per lung, ± s.e., 15.6 ± 9.7) and shEZH2 CNE2 cells (average number per lung, ± s.e., 2.9 ± 1.8). (**d**) Representative lungs, arrowheads (left) indicate metastatic nodules. Representative hematoxylin and eosin staining of lung metastatic tumors are shown (right).

These results provide further evidence that EZH2 negatively regulates E-cadherin expression in our NPC cells and that EZH2-mediated E-cadherin repression requires HDAC activity.

Transcription factor Snail is required for EZH2-mediated E-cadherin repression in NPC cells

To date, there is still little or no evidence that EZH2 could bind directly to the promoter sequences of *E-cadherin*. As Snail has been previously identified as a crucial transcriptional repressor of E-cadherin, by binding directly to the E-box of its promoter (Batlle *et al*., 2000; Cano *et al*., 2000), we investigated whether or not Snail is required for EZH2-mediated E-cadherin silencing. First, the basic levels of endogenous Snail in EZH2 low expressed 6-10B cells, shEZH2-HNE1 and shEZH2-CNE2 cells were knocked down by specific small interfering RNA (siRNA) and subsequently, the myc-EZH2 plasmid was transfected into these cells to check if EZH2 can still inhibit E-cadherin in the absence of Snail. As shown in Figure 3a, the repressive activity of EZH2 toward E-cadherin expression was virtually totally prevented in Snail depleted 6-10B, shEZH2-HNE1 and shEZH2-CNE2 cells. These data suggest that the presence of Snail is required and might be a predominant mediator for the repressive function of EZH2 toward E-cadherin.

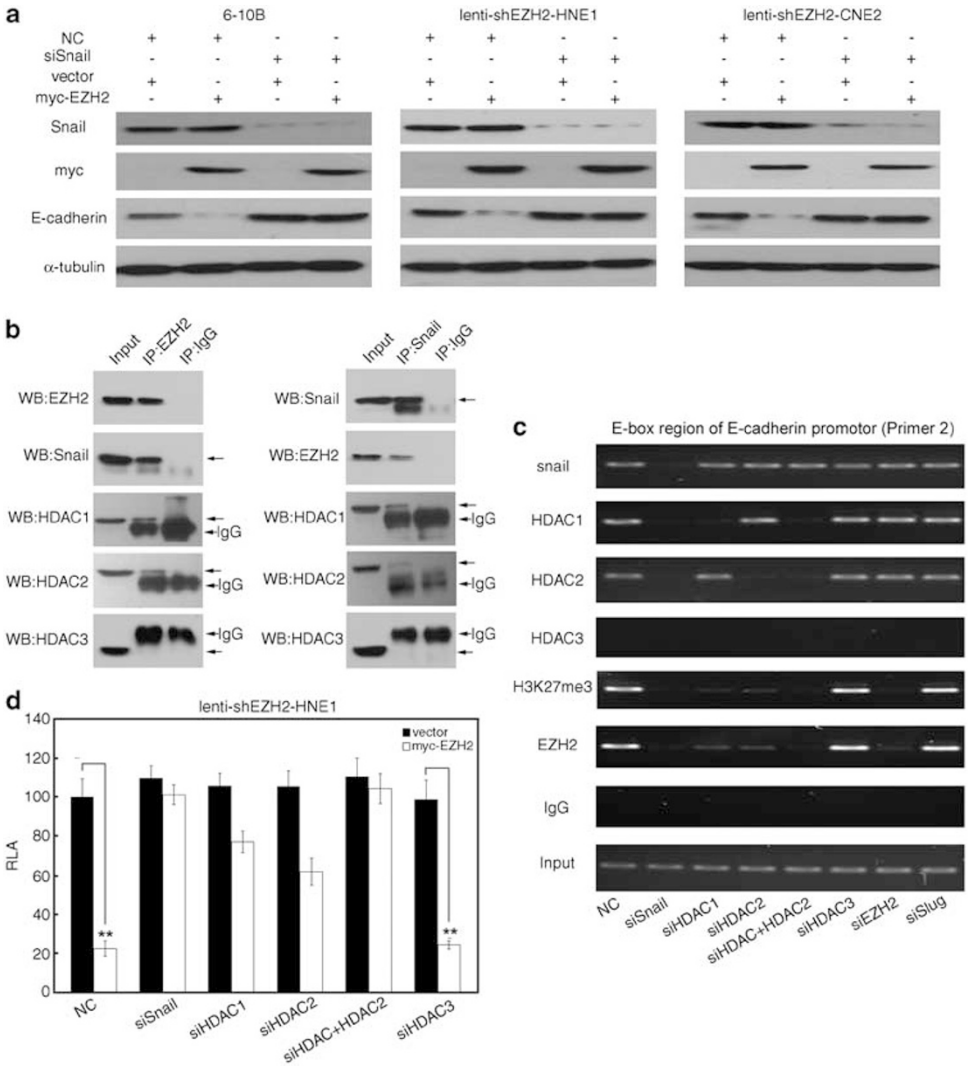

Figure 3 EZH2 silenced the expression of E-cadherin via assembling a co-repressor complex with HDAC1/2 and Snail. (**a**) The repression of E-cadherin by EZH2 is dependent on Snail in NPC cell lines. (**b**) The physical interaction between EZH2, HDAC1/2 and Snail examined by a co-IP assay. Arrows point to the bands representing indicated proteins. Results are representative data of three independent experiments. (**c**) HNE1 cells were transfected with a specific siRNA as indicated (bottom). After 72 h, ChIP was carried out to assess the enrichment of Snail, HDAC1/2/3, EZH2 and H3K27me3 in the E-box region of the *E-cadherin* promoter. (**d**) The lenti-shEZH2-HNE1 cells were transfected with specific siRNAs as indicated (bottom). After 48 h, the cells were co-transfected with the pGL3-*E-cadherin* promoter luciferase, a pRL-TK Renilla luciferase construct and a pIRES-EGFP (control) or a pIRES-EGFP-myc-EZH2 plasmid. After 36 h, luciferase activity of the *E-cadherin* promoter was measured and normalized relative to luciferase activity (RLA). The *P*-value was calculated by the Student's *t*-test. The bar shows the average ± s.d. of three independent experiments. $^{**}P<0.01$.

EZH2 interacted with HDAC1/HDAC2 and Snail to form a multi-molecular complex in NPC cells

As the above results provided evidence that the repressive function of EZH2 toward E-cadherin expression in NPC ells is dependent on HDACs activity and the presence of Snail, we wondered whether or not EZH2 might form a co-repressor complex with HDAC and/or Snail to repress E-cadherin in these cells. To address this issue, we performed a co-immunoprecipitation (IP) assay to test whether EZH2, HDACs and Snail could interact virtually in HNE1 NPC cells. As shown in Figure 3b (left), the existence of Snail, HDAC1 and HDAC2, but not HDAC3 was detected in the immunoprecipitates obtained with antibody against EZH2. Similarly, we detected EZH2, HDAC1 and HDAC2, but not HDAC3, in Snail immunoprecipitates (Figure 3b, right). Consistently, EZH2 and Snail was present in HDAC1 or HDAC2 immunoprecipitates, but was absent in HDAC3 immunoprecipitates (Supplementary Figure S1). These results indicate that EZH2 interacts with HDAC1/HDAC2 and Snail to form a multi-molecular complex.

The complex of EZH2/HDAC1/2/Snail contributed to E-cadherin silencing in NPC cells

To determine whether the EZH2/HDAC1/2/Snail complex could co-contribute to E-cadherin silencing in NPC cells, we further performed chromatin IP (ChIP) assays in HNE1 cells. The results indicated that EZH2, Snail, and HDAC1/HDAC2, but not HDAC3, strongly bind to the E-box region (that is, E-box1, E-box2 and E-box3, Supplementary Figure S2) of the *E-cadherin* promoter (Figure 3c). The depletion of Snail by siRNA dramatically dissociated HDAC1/HDAC2, EZH2 and H3k27me3 from the E-box region. However, knocking down of HDAC1 or HDAC2 by siRNA did not affect the binding of Snail, but markedly inhibited EZH2 recruitment to this region, concurrently with a decreased presence of H3K27me3; combined treatment with siHDAC1 and siHDAC2 enhances this kind of inhibition (Figure 3c). Treatment of HNE1 cells with siEZH2 caused a decreased enrichment of H3K27me3, while the recruitment of Snail and HDAC1/HDAC2 to the E-box region was not affected. In agreement with above IP results, we did not detect HDAC3 present on this region of *E-cadherin* promoter. In addition, we observed that the recruitment of HDAC1, HDAC2 and EZH2 to the E-box region was not affected by the knockdown of Slug (Snail 2), another member of the Snail protein family (Figure 3c). The knockdown efficiency of specific siRNAs targeted to the above genes is illustrated in Supplementary Figure S3a.

Subsequently, we used the luciferase reporter assay to test whether or not the repressive function of EZH2 on the E-cadherin promoter requires both Snail and HDAC1/HDAC2. Initially, the EZH2 stably silenced HNE1 cells were transfected with specific siRNAs targeted to Snail, HDAC1 and/or HDAC2, or HDAC3. Next, the cells were reintroduced with the myc-EZH2 plasmid and luciferase activity of the *E-cadherin* promoter was measured. As shown in Figure 3d, the repressive activity of ectopic EZH2 in the *E-cadherin* promoter of shEZH2-HNE1 cells was dramatically inhibited after the depletion of endogenous Snail, but was only partially inhibited after the depletion of either HDAC1 or HDAC2. With combined treatments with siHDAC1 and siHDAC2, this repressive activity was substantially enhanced compared with that of each alone. Collectively, these data indicate that EZH2, HDAC1/HDAC2 and Snail form a co-repressor complex to silence E-cadherin in NPC cells.

HDAC1/2-mediated EZH2 and Snail interaction in NPC cells

As our ChIP assay results indicated that knockdown of HDAC1/HDAC2 inhibits EZH2, but not Snail binding, to the *E-cadherin* promoter (Figure 3c), suggesting that HDAC1/HDAC2 might bridge the interaction between EZH2 and Snail, we then performed co-IP experiment again to verify this possibility. The results indicate that either siHDAC1 or siHDAC2 inhibits the interaction between EZH2 and Snail, and that the combined treatment with siHDAC1 and siHDAC2 abolishes this interaction (Figure 4a). Next, to determine whether the association of EZH2 and Snail is HDAC activity dependent, we treated HNE1 cells with three different gradient concentrations of TSA (HDAC inhibitor), and analyzed the amount of Snail in the EZH2 immunoprecipitates. As anticipated, we observed that TSA inhibited the interaction between EZH2 and Snail in a dose-dependent manner, and this interaction was virtually completely prevented when the cells were treated with 300 nM TSA (Figure 4b), while TSA treatment did not affect the protein levels of HDAC1, HDAC2, Snail and EZH2 (Supplementary Figure S3b). These results suggest that TSA inhibits the interaction between EZH2 and HDAC1/2 or between HDAC1/2 and Snail.

To determine which of these two interactions are affected by TSA, we performed co-IP experiments using antibodies anti-HDAC1 and anti-HDAC2. We found that 300 nM TSA significantly decreased the interaction between HDAC1/2 and Snail, but did not affect the association of HDAC1/2 and EZH2 (Figure 4c). Consistently, the ChIP assay showed that when HNE1 cells were treated with 300 nM TSA, the binding of Snail to *E-cadherin* was not affected, but the recruitment of HDAC1, HDAC2 and EZH2 to the *E-cadherin* promoter was substantially suppressed, and, in addition, the levels of H3K27me3 were reduced (Figure 4d). Also, the luciferase reporter assay supported these results, that is, the repressive activity of ectopic EZH2 on the *E-cadherin* promoter was blocked in the shEZH2-HNE1 cells pretreated with 300nM TSA, in contrast to that of controls (Figure 4e). These observations, taken together, provide substantial evidence that in NPC cells, Snail, HDAC1/2 and EZH2 formed a multi-molecular complex, and that the interaction between Snail and EZH2 is bridged by HDAC1/2.

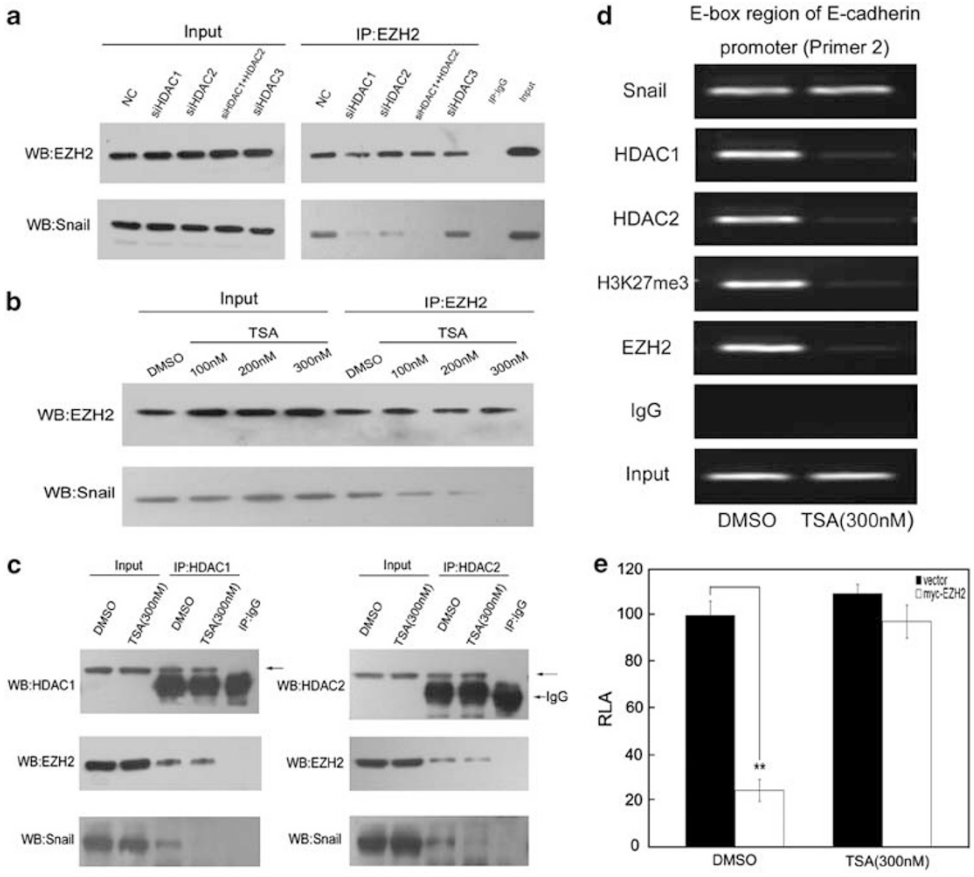

Figure 4 HDAC1/2 mediated the interaction between EZH2 and Snail. (**a**) Silencing of HDAC1 or HDAC2 inhibited the affinity between Snail and EZH2. HNE1 cells were transfected with a specific siRNA as indicated. After 72 h, cell extracts were subjected to IP with anti-EZH2, and a western blot was performed with antibodies against EZH2 and Snail. One percent of the whole cell lysate was prepared for precipitation and served as a control. (**b**) TSA was sufficient to decrease an interaction between EZH2 and Snail in a dose-dependent manner. HNE1 cells were treated with the vehicle and three different concentrations of TSA, for 48 h, and then the cell lysate was subjected to co-IP assays. (**c**) TSA inhibited the interaction between HDAC1/2 and Snail but did not influence the interaction between HDAC1/2 and EZH2. HNE1 cells were treated with the vehicle or TSA (300 nM). After 48 h, cell extracts were subjected to IP with anti-HDAC1 or anti-HDAC2, and the presence of EZH2 and Snail protein levels in the precipitates were assessed by western blot. (**d**) The binding levels of Snail, EZH2 and HDAC1/2 and abundance of H3K27me3 in the E-box region of the *E-cadherin* promoter were determined by ChIP in TSA (300 nM) or DMSO treated HNE1 cells. (**e**) The lenti-shEZH2-HNE1 cells were pretreated with 300 nM TSA for 48 h, then co-transfected with pGL3-E-cadherin promoter luciferase, pRL-TK Renilla luciferase construct and pIRES-EGFP or pIRES-EGFP-myc-EZH2 plasmid. After 36 h, luciferase activity of the *E-cadherin* promoter was measured and normalized as RLA. $^{**}P<0.01$.

The Snail, but not Yin-Yang (YY) 1, contributes to the recruitment of EZH2 and HDAC1/2 to the promoter of E-cadherin

Recently, evidence has been reported that the mammalian protein YY1 may contribute to the process of PRC2 recruitment to the promoter region of different target genes (Satijn *et al*., 2001; Schwartz and Pirrotta, 2007; Simon and Kingston, 2009), and that YY1 is needed for H3k27 methylation of target genes in muscle cells (Caretti *et al*., 2004). Thus, we proposed that besides Snail, YY1 may also participate in the recruitment of EZH2, to the *E-cadherin* promoter. We designed a primer (primer 1), whose products contained three predicted YY1-binding sites, but do not contain E-box sequences (Supplementary Figure S2), to perform ChIP assay. The results showed that Snail, but not YY1, mediated EZH2 and HDAC1/2 recruiting to the *E-cadherin* promoter (Figures 5a–c and Supplementary Figure S4).

Discussion

It has been proposed that the gene, *EZH2*, could be a novel candidate oncogene and/or therapeutic target for several types of human cancers (Varambally *et al*., 2002; Raman *et al*., 2005; Chen *et al*., 2007; Simon and Lange, 2008). In this study, a potential relationship between EZH2 and the oncogenicity of NPC was investigated. Western blot and IHC staining demonstrated that high expression of EZH2 was frequently detected in our NPC tissues. Further correlation analysis provided evidence that high expression of EZH2 in our NPC cohorts was positively correlated with an advanced clinical stage and was a strong and independent predictor of poor patient survival. These findings, taken together, underscore a potentially important role of *EZH2* as an underlying biological mechanism in the development and/or progression of NPC.

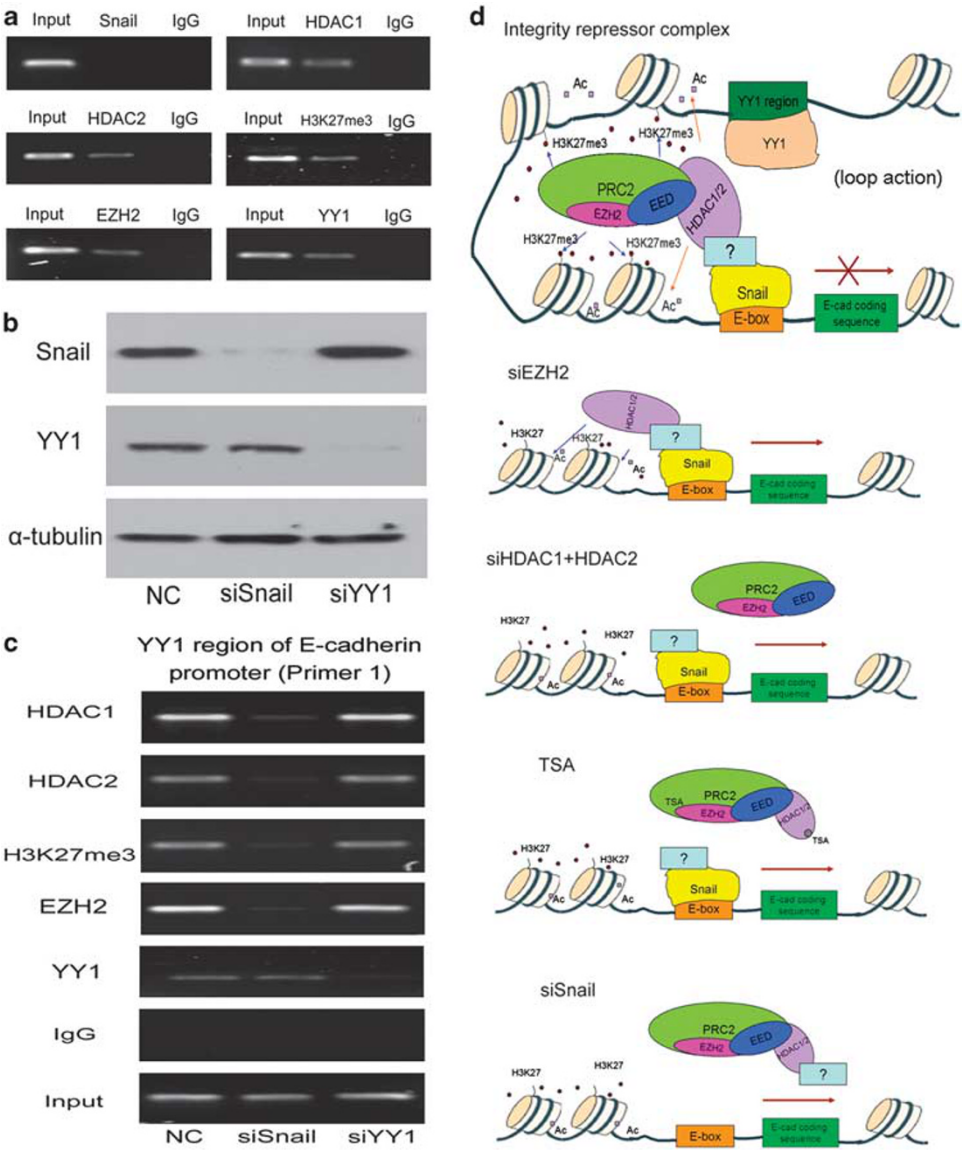

Figure 5 The Snail, but not YY 1, contributes to the recruitment of EZH2 and HDAC1/2 to the promoter of *E-cadherin* (**a**) ChIP assay was carried out on HNE1 cells to identify the presence of Snail, HDAC1/2, EZH2 and H3K27me3 in the YY1 region of the *E-cadherin* promoter. The results showed that except for Snail, EZH2, HDAC1/2, H3K27me3 and YY1 were all present in the YY1 region of the *E-cadherin* promoter. (**b**) Knock down efficiency and specificity of siSnail and siYY1 was evaluated by Western blot. (**c**) HNE1 cells were pretreated with a siRNA target on Snail or YY1 for 72 h. Subsequently, ChIP assays were performed to determine the enrichment of HDAC1/2, YY1, EZH2 and H3K27me3 in the YY1 region of the *E-cadherin* promoter. When Snail was knocked down by siRNA, the proteins EZH2 and HDAC1/2 were all dissociated from the YY1 binding region concomitant with a reduced presence of H3K27me3. The inhibition of endogenous YY1 by siRNA did not influence the binding of EZH2, HDAC1/2 or H3K27me3 in this region. (**d**) Proposed schematic representation of a molecular mechanism of Snail, HDAC1/2 and EZH2 in the suppression of E-cadherin.

Recently, we have reported that EZH2 supported ovarian carcinoma cell invasion and/or metastasis via regulation of TGF-β1 (Rao *et al*., 2010). Other reports have provided evidence that EZH2 contributed to cancer cell invasion and metastasis by downregulation of the tumor-suppressor E-cadherin through trimethylation of H3K27 (Matsukawa *et al*., 2006; Cao *et al*., 2008). In this study, we observed that NPC patients with a high expression of EZH2 had a decreased expression of E-cadherin, but was not associated with expression of TGF-β1 (data not shown). In addition, ectopic overexpression of EZH2 in NPC cells downregulated E-cadherin expression that was accompanied with an increased H3K27me3. In accord, knocking down of EZH2 in NPC cells caused an increased expression of E-cadherin associated with downregulated H3K27me3 levels. These data suggest that EZH2 negatively regulates E-cadherin expression in NPC cells via trimethylation of H3K27. To date, however, the precise mechanism by which EZH2 regulates E-cadherin expression remains unclear.

In a recent study of ours using NPC cells, another PRC member, Bmi-1, was found to transcriptionally

decrease the expression levels of the phosphatidylinositol 3 kinase (PI3K)/AKT pathway suppressor phosphatase and tensin homologue deleted on chromosome 10 (PTEN), through a direct association with the PTEN locus. Such caused the stabilization of Snail via activation of PI3K/AKT signaling and ultimately inhibited E-cadherin expression; EZH2 was also detected at the PTEN promoter locus (Song *et al*., 2009). In addition, the repressive function of PRC2 is required for PRC1 recruitment to target genes (Cao *et al*., 2002; Boyer *et al*., 2006; Simon and Lange, 2008), and PRC2 inactivation by Suz12 siRNA was found to increase mRNA levels of *PTEN* (Herranz *et al*., 2008; Song *et al*., 2009). These results suggest that EZH2 might also downregulate PTEN and consequently involve the PI3K/AKT/Snail pathway to repress E-cadherin. In this study, however, we did not examine the altered levels of PTEN, phosphorylated AKT, AKT and Snail before and after EZH2 knocking down (Supplementary Figure S5). It does appear, therefore, that in our NPC cells, the repression of E-cadherin by EZH2 is via pathways other than the PTEN/PI3K/AKT/Snail pathway.

To our knowledge, up to date, none of PRC2 core components were possessed the sequence-specific DNA-binding activity, the exact mechanism of EZH2 recruiting to target gene is still unrevealed (Muller and Kassis, 2006; Wilkinson *et al*., 2010). Recently, Herranz *et al*. (2008) found that zinc-finger factor Snail could recruit PRC2 complex to the *E-cadherin* promoter (Herranz *et al*., 2008). In this study, we found that the repressive activity of EZH2 in E-cadherin expression was virtually completely prevented, when Snail was knocked down in NPC cells. These data suggest that the presence of Snail is required for the repressive function of EZH2 toward the *E-cadherin*. Additionally, we observed that in NPC cells, the repressive function of EZH2 toward the *E-cadherin* was dependent on HDAC activity. Other groups previously reported that Snail mediates E-cadherin repression by the recruitment of the Sin3A/HDAC1/2 complex (Peinado *et al*., 2004), and E-cadherin is suppressed by a Snail/HDAC1/HDAC2 complex to regulate metastasis of pancreatic cancer *in vivo* (von Burstin *et al*., 2009). These results, taken together, prompted us to ask (1) whether or not EZH2, the core member of PRC2, acts as a co-repressor complex with HDAC1/2 and Snail to repress E-cadherin in NPC cells, and (2) if so, in which fashion does these components interact each other. To address these issues, further co-IP, ChIP and luciferase reporter assays were performed. Our results provide evidence that in NPC cells, (1) EZH2 interacted with HDAC1/HDAC2 and Snail, but not HDAC3, to form a repressive complex; (2) these components interact in a linear fashion, not in a 'triangular' fashion, that is, HDAC1 or HDAC2 bridge the interaction between EZH2 and Snail; and (3) the EZH2/HDAC1/2/Snail complex could closely bind to the *E-cadherin* promoter by Snail, but not YY1, to repress E-cadherin. Our current data were limited to clarify whether these proteins interact directly or indirectly. It had been revealed that HDAC2 could interact directly with EED (another component of PRC2), and indirectly with EZH2 (van der Vlag and Otte, 1999), suggesting that the interaction between EZH2 and HDAC1/2 is mediated by EED. However, the exact binding fashion between HDAC1/2 and Snail is still undetermined. In addition, it is noteworthy here, we found that HDACs inhibitor TSA remarkably suppressed the interaction between Snail and HDAC1/2 without affecting their protein levels, and consequently dissociated EZH2 and HDAC1/2 from the promoter of *E-cadherin*. Owing to the fact that the previous crystal structure study revealed that TSA could bind to HDLP (HDAC homologue) by inserting its long aliphatic chain into enzymatic activity pocket, causing multiple contacts to the tube-like hydrophobic portion of the pocket (Finnin *et al*., 1999), it is possible that TSA inhibited the interaction between HDAC1/2 and Snail via altering the conformation of their binding regions, not by steric hindrance effect.

In *Drosophila*, polycomb response elements have been identified using reporter assays and several DNA-binding proteins are implicated in recruiting PRC2 (Simon and Lange, 2008). To date, however, mammalian polycomb response elements have not been defined. In accord to previous results, a good candidate recruiter of PRC2 is YY1 (Wang *et al*., 2004; Simon and Lange, 2008). Indeed, YY1 is needed for H3K27me3 of target genes in muscle cells (Caretti *et al*., 2004). Thus, we further investigated whether YY1 can contribute to recruiting EZH2 to *E-cadherin* promoter. Intriguingly, we observed that YY1 was present in the YY1 region of *E-cadherin* promoter. But our ChIP assay showed that siSnail, but not siYY1, dissociated the recruitment of EZH2, HDAC1/2 from this region. Several groups had reported that the PcG proteins and H3K27me3 were tend to be broadly distributed across many kilobases of target gene locus (Boyer *et al*., 2006; Lee *et al*., 2006; Schwartz *et al*., 2006; Simon and Kingston, 2009). An alternative interpretation is that the large distribution of PcG complex is account for the looping action mechanism, leading PcG complex to be crosslinked to more distant nucleosomes (Schwartz and Pirrotta, 2007; Tiwari *et al*., 2008). Therefore, it is not difficult to understand why siSnail, but not siYY1 dissociated EZH2 and HDAC1/2 from the YY1 region of *E-cadherin* promoter. As a result of our collective present data, together with other group's findings (van der Vlag and Otte, 1999; Peinado *et al*., 2004; Herranz *et al*., 2008; von Burstin *et al*., 2009), herein we propose a main co-repressor complex, that is, EZH2/HDAC1/2/Snail, in the regulation of E-cadherin in NPC cells, and probably in a number of other types of human cancers. A schematic representation of the major molecular mechanisms of this complex, in NPC cells, is suggested and provided in Figure 5d. Clearly, further works are needed to confirm this model and elucidate how these components interacts each other in detail.

In summary, our study describes the expression pattern of EZH2 in the human NPC tissues. Furthermore, our results provide a basis for the concept that upregulated expression of EZH2 in NPC may be important in the acquisition of an aggressive/poor

prognostic phenotype. Our results provide functional and mechanistic links between the oncoprotein EZH2 and the tumor-suppressor E-cadherin in the aggressive nature of NPC.

Materials and methods

Cell culture, antibodies and chemical reagents
See Supplementary Materials and methods for detail.

NPC patients and tissue microarray
In all, 209 specimens of NPC and 50 specimens of non-neoplastic nasopharyngeal mucosa were collected at Sun Yat-Sen University Cancer Center and Guangdong Provincial People's Hospital, Guangzhou, China, between January 1991 and August 2000. A NPC–tissue microarray was constructed as previously described (Xie *et al*., 2003). Clinico-pathological characteristics of the NPC patients are summarized in Table 1. In addition, 13 pairs of fresh NPC tissue and adjacent non-neoplastic nasopharyngeal mucosa specimens were collected in 2009. None of the NPC patients had received preoperative radiation or chemotherapy before diagnosis. This study was approved by the medical ethics committee of our institutes.

IHC staining and western blot
See Supplementary Materials and methods for detail.

Fluorescence in situ *hybridization*
The fluorescence *in situ* hybridization reaction was performed as described previously (He *et al*., 2010).

Knocking down of EZH2 by lentiviral shRNA
See Supplementary Materials and methods for detail.

Mammalian expression plasmids construction and transfection
Myc-EZH2 fragment was amplified by PCR and cloned into a pIRES2-EGFP vector (Clontech, Palo Alto, CA, USA). The SET domain deletion mutation of EZH2 (myc-EZH2ΔSET) was generated by PCR according to a Quikchange Site-Directed Mutagensis Kit (Stratagene, La Jolla, CA, USA) instruction manual. The primer used for this deletion mutation is 5′-GTGT CCTGCAAGAACTGCAGTCAGGCTGATGCCCTGAAG-3′. The expression plasmid was transfected into cells by a Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer's instructions.

Matrigel invasion assays
See Supplementary Materials and methods for detail.

Experimental in vivo *metastasis assay*
Tumor metastatic ability of EZH2 shRNA NPC cells were determined by tail vein intravenous injection into 6-week-old Balb/C athymic nude mice (nu/nu, Slac Laboratory Animal Co. Ltd, Shanghai, China). Briefly, each experiment group consisted of nine mice. Control cells, lenti-Luc-CNE2 cells and lenti-shEZH2-CNE2 cells were injected intravenously through the tail vein into each mouse (5×10^5 cells per mouse). All mice were killed by cervical dislocation 2 months later, and the number of tumor nodules formed in the lung was counted and analyzed by hematoxylin and eosin staining.

Depletion of Snail, HDAC1/2/3, EZH2 and Slug by synthetic siRNA
See Supplementary Materials and methods for detail.

ChIP and IP assays
See Supplementary Materials and methods for detail.

Dual luciferase reporter assay
Dual luciferase reporter assay was performed as described previously (Cao *et al*., 2008) with slight modification. Briefly, the treated lenti-shEZH2-HNE1 cells were lysed, and luciferase and renilla activities were analyzed using a dual luciferase assay kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. All reporter gene assays were performed in triplicate and repeated twice. The results were expressed as the mean ± s.e.

Statistical analysis
Statistical analysis was performed using a SPSS software package (SPSS Standard version 13.0, SPSS Inc., Chicago, IL, USA). Differences between variables were assessed by the χ^2 test or Fisher's exact test. For survival analysis, an optimal cut-point for IHC EZH2 expression was selected using the X-tile software version 3.6.1 (Yale University School of Medicine), as described previously (Camp *et al*., 2004). Data derived from cell line experiments are presented as mean ± s.e. ($X \pm$ s.e.) and assessed by the two-tailed Student's *t*-test. *P*-values of <0.05 were considered statistically significant.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)