



## The polycomb group protein EZH2 inhibits lung cancer cell growth by repressing the transcription factor Nrf2



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### ABSTRACT

**EZH2 is a key component of the polycomb PRC2 complex and functions as a histone H3 Lys27 (H3K27) trimethyltransferase. Here we show that EZH2 is down-regulated in human non-small cell lung cancer and low EZH2 expression predicts poor survival. Further we demonstrate that EZH2 inhibits lung cancer cell proliferation and colony formation in vitro and growth in vivo. We found that EZH2 binds to the promoter of Nrf2, where it increases H3K27me3 and represses Nrf2 expression. Finally, Nrf2 seems to be essential for the hyper proliferation of lung cancer cells in the absence of EZH2.**

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### 1. Introduction

Post-translational modification of histones by methylation is an important and widespread type of chromatin modification that is known to influence biological processes in the context of development and cellular responses [1]. Histone lysine methylation is a critical player in the regulation of gene expression, cell cycle, genome stability, and nuclear architecture [2]. Lysines can be monomethylated (me1), dimethylated (me2) or trimethylated (me3) on their  $\epsilon$ -amine groups [1]. The most extensively studied histone methylation sites include histone H3 lysine 4 (H3K4), H3K9, H3K27, H3K36, H3K79 and H4K20 [1]. Trimethylation of histone H3 lysine 27 (H3K27me3), catalyzed by the PcG enhancer of zester homolog 2 (EZH2), is associated with transcriptional repression; whereas H3K4 methylation, catalyzed by the trithorax homolog myeloid-lymphoid leukemia (MLL), is associated with transcriptional activation [3]. Importantly, many genes involved in development, stem cell maintenance, and differentiation are targets of H3K27 and H3K4 methylation. Alteration of histone methyl

modifiers and methyl-binding proteins correlates with increased incidence of various cancers [4,5]. One typical example is the H3K27 methyltransferase EZH2. EZH2 is up-regulated in a number of cancers, including prostate cancer [6], breast cancer [7], and lymphomas [8].

Lung cancer is one of the leading causes of cancer-related death in developed countries. Despite decades of intensive efforts, the prognosis of lung cancer remains unfavorable and is especially poor in advanced non-small cell lung cancer (NSCLC). One of the main causes of the poor outcome in NSCLC treatment is the innate resistance to anticancer drugs. The transcription factor NF-E2-related factor 2 (Nrf2) is a basic-leucine-zipper transcription factor originally identified as a pivotal factor for protecting cells from oxidative and electrophilic insults [9]. Nrf2 effector genes include a majority of anti-oxidative, phase II detoxifying enzymes, and ATP-dependent drug efflux pumps [10,11]. Activation of Nrf2 in cancer cells provides advantages for cell proliferation and survival from exposure to anticancer drugs [12,13]. Several studies have implicated the role of Nrf2 in tumor growth and drug-resistance in NSCLC [11,14–17]. Our recent work showed that hMOF acetylates Nrf2 and promotes its nucleus retention in human NSCLC [18]. However, how *Nrf2* is controlled at the transcription level remains unknown.

Here in this study, we report that EZH2 represses NSCLC progress by epigenetically inhibiting *Nrf2* expression.

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## 2. Materials and methods

### 2.1. Patients

54 patients with NSCLC were included in this study. The patients were recruited at Shanghai Lung Tumor Clinical Medical Center, Shanghai Chest Hospital, Shanghai Jiao Tong University. The 11 adjacent normal lung tissues were obtained from NSCLC patients undergoing surgery. The clinical characteristics of the patients is listed in [Table 1](#). The diagnosis of lung cancer was established using World Health Organization (WHO) morphological criteria. Both the adjacent normal tissues and the lung cancer tissues were confirmed by a pathologist. Clinicopathological parameters including age, gender, smoking history, histologic grade, tumor status, lymph node metastasis and histologic type were analyzed. A written form of informed consent was obtained from all patients, and the study was approved by the Clinical Research Ethics Committee of Shanghai Jiaotong University.

### 2.2. Cells and cell culture

Human lung cancer cell line A549 was obtained from Riken Bio-Resource. The cells were cultured in high glucose-containing DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin.

### 2.3. Quantitative real-time PCR (q-PCR)

Total RNA samples were extracted from tissues or cells with TRIzol reagent (TaKaRa). cDNA was synthesized from 2 µg of RNA with One Step RT-PCR Kit (TaKaRa). q-PCR was performed with the SYBR Green (TaKaRa) detection method on an ABI-7500 RT-PCR system (Applied Biosystems). The EZH2 primers (NM\_004456), Nrf2 primers (NM\_006164), NQO-1 primers (NM\_000903), and HO-1 primers (NM\_002133) were purchased from OriGene Technology.

**Table 1**  
Clinical characteristics of the patients with NSCLC.

Variables	Total	EZH2 low expression (26)	EZH2 high expression (28)	<i>p</i> value
<b>Age</b>				
≥60	32	17	15	0.418
<60	22	9	13	
<b>Gender</b>				
Male	34	15	19	0.4396
Female	20	11	9	
<b>Smoking history</b>				
Never	19	7	12	0.0205
Ever	35	25	10	
<b>Histology type</b>				
Adenocarcinomas	26	15	11	0.2756
Squamous cell carcinomas	28	11	17	
<b>Tumor size (cm)</b>				
≥3	30	20	10	0.003
<3	24	6	18	
<b>Metastasis and recurrence</b>				
Present	21	15	6	0.0113
Absent	33	11	22	
<b>Stage</b>				
I	8	1	10	0.0056
II	17	6	8	
III	29	19	10	

### 2.4. Western blot

Tissues and lung cancer cells were lysed with cell lysis buffer (Beyotime) supplemented with protease inhibitor cocktail. 40 µg total proteins were applied to 12% SDS–polyacrylamide gel. After electrophoresis, the proteins were transferred to PVDF membranes, followed by blocking in the buffer containing 5% fat-free dry milk. The membranes were then probed with indicated antibodies overnight, and then washed and incubated with HRP-conjugated secondary antibodies (Zhongsanjinqiao) for 2 h and finally visualized using Chemiluminescent ECL reagent (Vigorous Biotechnology). The following antibodies were used: Anti-GAPDH (Cell Signaling Technology, #2118), anti-EZH2 (Cell Signaling Technology, #4905), anti-Nrf2 (Abcam, #ab31163), anti-H3K27me3 (Abcam, #ab6002), anti-Histone H3 (Abcam, #ab1791).

### 2.5. Adenovirus/retrovirus packaging and retroviral transduction

To prepare EZH2-expressing adenovirus, the human EZH2 cDNA was inserted into D-TOPO vector (Invitrogen). The D-TOPO-EZH2 plasmid was cloned into the pAd/CMV/V5-DEST vector (Invitrogen) using LR Clonase (Invitrogen). The plasmid was linearized with PacI and was transfected into 293A cells for production of adenovirus. For Ad-Ctrl, the GFP cDNA was inserted into D-TOPO vector instead of EZH2 cDNA. Control shRNA and specific shRNAs targeting EZH2 or Nrf2 were purchased from Invitrogen, and the corresponding sequences were cloned into the pSIREN-RetroQ plasmid (Addgene) for retrovirus production with 293T cells. The targeting sequences were listed in [Supplementary Table 1](#).

For transduction, 293T cells were incubated with virus-containing supernatant in the presence of 8 mg/ml polybrene. After 48 h, infected cells were selected for with puromycin (2 mg/ml). Then the clones were picked and cultured for further experiment.

### 2.6. Chromatin immunoprecipitation (ChIP)

ChIP was carried out using the ChIP-IT Express Enzymatic kit (Active Motif) according to the manufacturer's instructions. In brief, chromatin from cells was cross-linked with 1% formaldehyde (10 min at 22 °C), sheared to an average size of ~500 bp and then immunoprecipitated with anti-EZH2, anti-IgG or anti-H3K27me3 antibodies. q-PCR was performed to test the binding ability of EZH2 to *Nrf2* promoter regions. The detailed information on EZH2 binding regions and the primers were given in [Supplementary Fig. 1 and Table 2](#).

### 2.7. Cell proliferation assay

Cell proliferation was monitored by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation/Viability Assay kit (R&D SYSTEMS) in according to the guidelines.

### 2.8. Soft sugar colony formation assay

Soft sugar colony formation assay was performed as described previously [19]. Cell colonies were stained with 0.005% crystal violet and analyzed using a microscope. The colony number in each well was calculated.

### 2.9. Luciferase assay

We designed the luciferase assay in according to the results of ChIP assay, and the region where EZH2 binds most frequently was chose for luciferase assay. In detail, the human Nrf2 (–750 to –0 bp) promoter was cloned into the pGL4 reporter vector (Promega) to generate an Nrf2-luc reporter vector. 293T cells were

cultured in triplicate in 24-well plates until reaching 80% confluence. The cells were then co-transfected with Nrf2-Luc/p-RL-luc and EZH2 expressing or control vectors as indicated in the figure legend. Dual luciferase assays (Promega) were performed as described by the manufacturer.

### 2.10. Xenograft mice experiment

Xenograft mice experiments were performed as described previously [20]. In brief, equal numbers of A549 cells expressing either control or EZH2 knockdown/overexpression vectors were injected subcutaneously, within 30 min of harvesting, over the right and left flanks in male nu/nu mice between 4 and 6 weeks of age. Tumor growth was monitored using calipers. The mice were killed and the tumors were weighted 3 weeks after inoculation.  $N = 10$  in each group.

### 2.11. Statistical analysis

All values are expressed as the means  $\pm$  S.E.M. At least three replicates were applied for each experiments if no additional information was indicated in this study. Statistical differences among groups were determined using either Student's *t* test or one-way ANOVA. The correlation of EZH2 mRNA level with patients' clinicopathological variables was analyzed by the  $\chi^2$  test or Fisher's exact test. *P* values of less than 0.05 were considered statistically significant.

## 3. Results

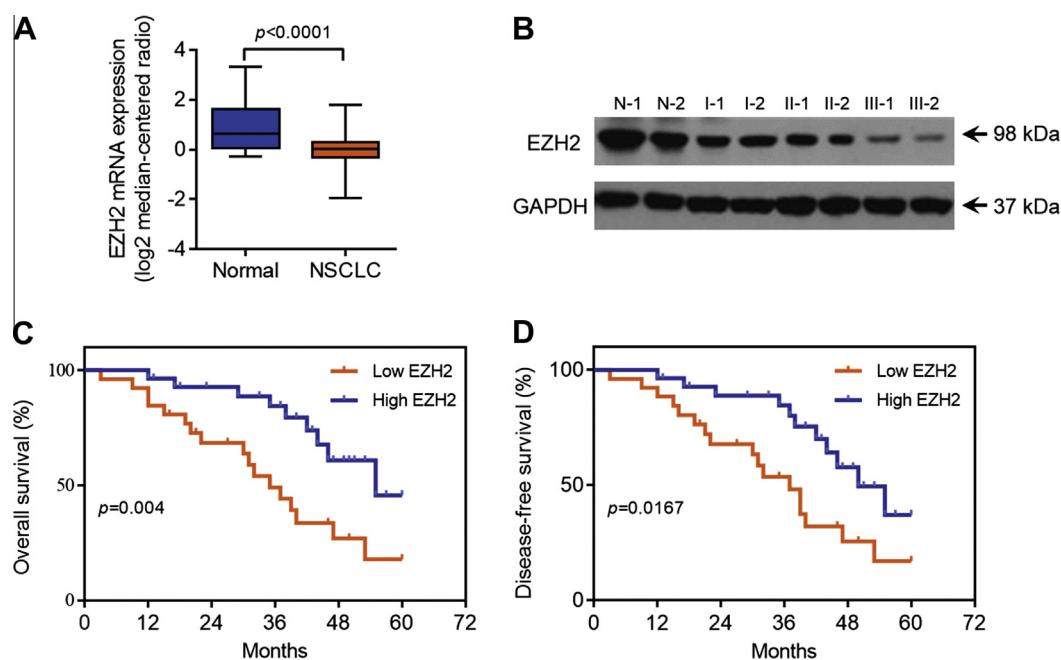
### 3.1. EZH2 is decreased in NSCLC and low EZH2 level predicts poor survival

To investigate EZH2 function in human NSCLC, we examined changes in EZH2 mRNA and protein expression in human NSCLC

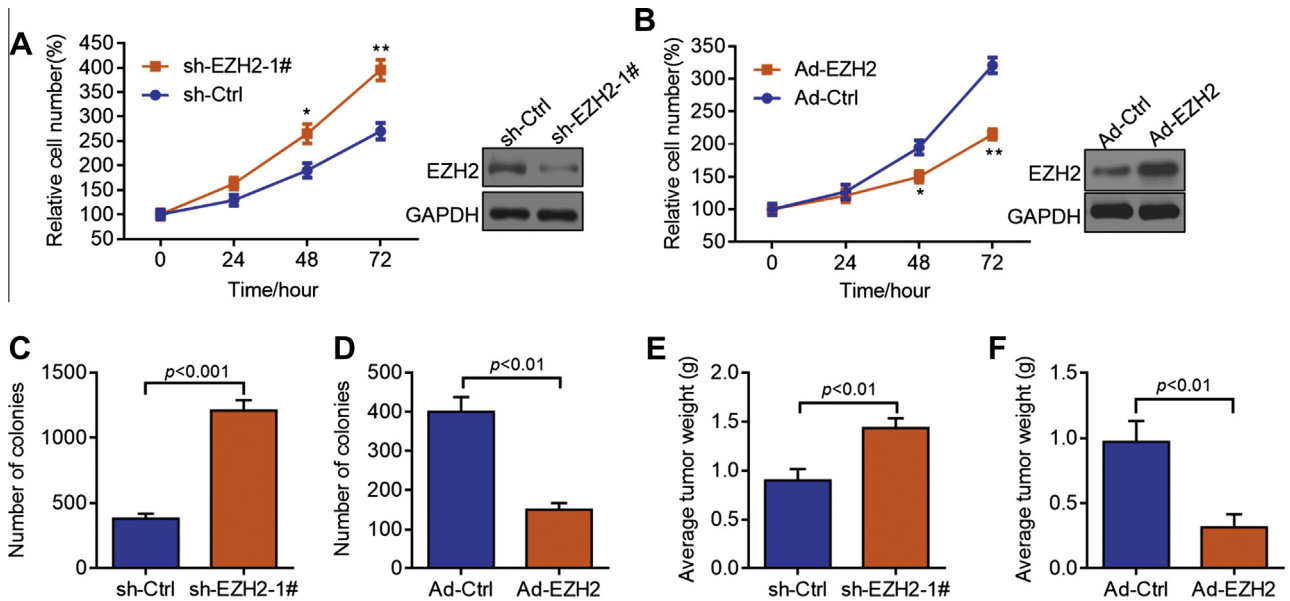
and adjacent normal tissues. The results showed that EZH2 expression was significantly down-regulated in NSCLC tissues at mRNA and protein levels (Fig. 1A and B), and EZH2 level decreased with the development of cancer (Fig. 1B). Those data implicates that EZH2 may participate in the development and prognosis of NSCLC. To test this hypothesis, we analyzed the correlation between mRNA level of EZH2 and overall and disease-free survival in patients with NSCLC. Markedly, low expression of EZH2 was correlated with poor overall and disease-free survival (Fig. 1C and D). In addition, low EZH2 level was correlated with large tumor size, high metastasis, and high disease stage (Table 1). Those results indicate that EZH2 expression is down-regulated in human NSCLC tissues and that low level of EZH2 predicts poor survival.

### 3.2. EZH2 regulates lung cancer cell growth in vitro and in vivo

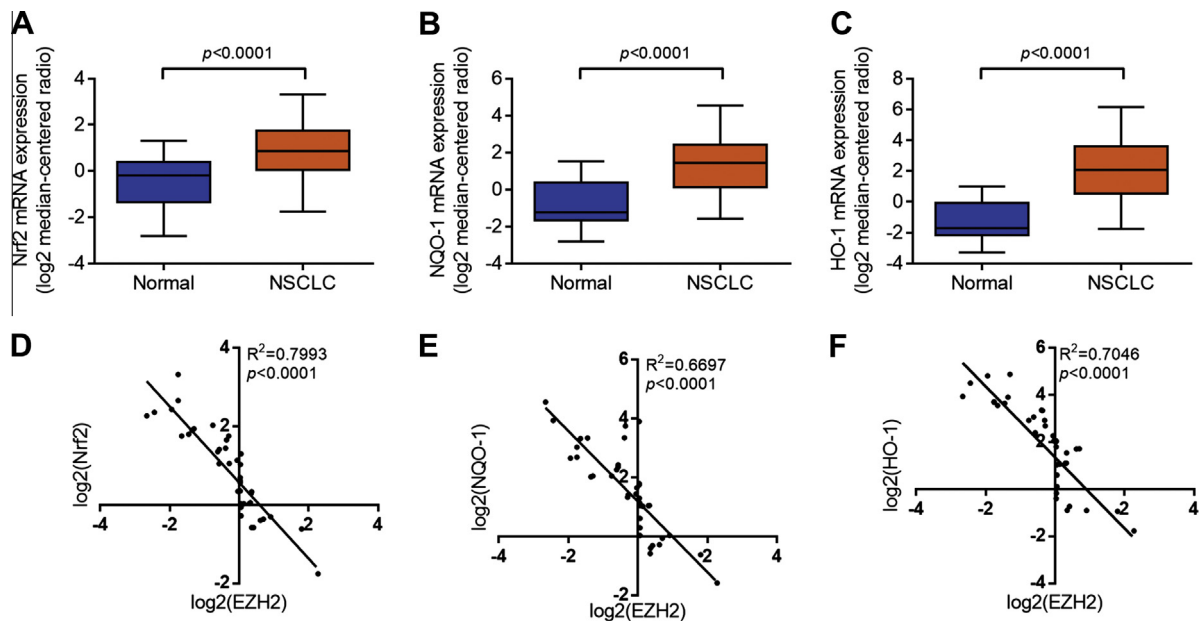
To explore the role of EZH2 in lung cancer cell growth, we performed cellular proliferation and colony formation experiments in vitro and xenograft mice experiments in vivo. We knocked down EZH2 expression in A549 lung cancer cells and found that EZH2 knockdown promoted A549 cell proliferation (Fig. 2A, Supplementary Fig. 1A and B). In contrast, when EZH2 was overexpressed, the proliferation rate of A549 cells decreased significantly (Fig. 2B). Next, we evaluated the colony formation capacity. The results showed that EZH2 knockdown up-regulated colony formation activity of A549 cells (Fig. 2C, Supplementary Fig. 1C, D and 2A), while EZH2 overexpression reduced colony formation activity of A549 cells (Fig. 2D, Supplementary Fig. 2B). Those results indicate that EZH2 inhibits lung cancer growth in vitro, which prompted us to confirm the role of EZH2 in lung cancer in vivo. Therefore, we performed xenograft mice experiments. Our results showed that EZH2 knockdown increased the average tumor weight (Fig. 2E, Supplementary Fig. 3A), whereas EZH2 overexpression decreased the average tumor weight of lung cancer (Fig. 2F, Supplementary Fig. 3B). Taken together, our findings demonstrate that EZH2 inhibits lung cancer growth in vitro and in vivo.



**Fig. 1.** EZH2 expression decreases in NSCLC and low EZH2 level predicts poor survival. (A) EZH2 mRNA level is decreased in human NSCLC tissues. EZH2 mRNA levels were determined with q-PCR in adjacent normal lung tissues versus lung cancer tissues. The boxes represent the interquartile range; whiskers represent the 10th–90th percentile range; bars represent the median.  $N = 11$  in normal group;  $n = 54$  in NSCLC group. (B) EZH2 protein level is decreased in human NSCLC tissues. Protein was extracted from adjacent normal lung tissues and lung cancer tissues from different stages, and the protein was subjected to Western blot analysis with indicated antibodies. N: Normal lung tissues; I: Stage I; II: Stage II; III: Stage III. (C, D) Kaplan–Meier curve comparing time to survival between NSCLCs with the low (<25th percentile) versus high (>25th percentile) EZH2 expression was determined using the q-PCR results. (C) Overall survival. (D) Disease-free survival.



**Fig. 2.** EZH2 expression regulates lung cancer growth in vitro and in vivo. (A) EZH2 knockdown promotes A549 lung cancer cell proliferation. A549 lung cancer cells were infected with retrovirus expressing ctrl shRNA (sh-Ctrl) or shRNA targeting EZH2 (sh-EZH2-1#). The relative cell numbers were evaluated with MTT method at the indicated time points. (B) EZH2 overexpression inhibits A549 lung cancer cell proliferation. A549 lung cancer cells were infected with adenovirus expressing GFP (Ad-Ctrl) or EZH2 (Ad-EZH2). The relative cell numbers were evaluated at the indicated time points. (C) EZH2 knockdown promotes A549 lung cancer cell colony formation. A549 cells infected with retro-sh-Ctrl or retro-sh-EZH2-1# were subjected to soft sugar colony formation assay. The colony numbers were evaluated two weeks later. (D) EZH2 overexpression inhibits A549 lung cancer cell colony formation. A549 cells infected with Ad-Ctrl or Ad-EZH2 were subjected to soft sugar colony formation assay. The colony numbers were evaluated two weeks later. (E, F) A549 cells with EZH2 knockdown or overexpression were subjected to xenograft mice experiments. Tumor weight was evaluated at the end of the experiments. (E) EZH2 knockdown facilitates lung cancer cell growth in vivo. (F) EZH2 overexpression inhibits lung cancer cell growth in vivo. \* $P < 0.05$ ; \*\* $P < 0.01$ .



**Fig. 3.** EZH2 is correlated with the expression of Nrf2 and its downstream genes. (A–C) Nrf2, NQO-1 and HO-1 mRNA levels were determined in adjacent normal lung tissues versus lung cancer tissues. The boxes represent the interquartile range; whiskers represent the 10th–90th percentile range; bars represent the median. (A) Nrf2 mRNA is increased in lung cancer tissues. (B) NQO-1 mRNA level is increased in lung cancer tissues. (C) HO-1 mRNA level is increased in lung cancer tissues.  $N = 11$  in normal group;  $n = 44$  in NSCLC group. (D–F) Linear regression analysis was performed to analyze the correlation between EZH2 expression and Nrf2, NQO-1 or HO-1 expression. (D) EZH2 mRNA level is negatively correlated with the level of Nrf2. (E) EZH2 mRNA level is negatively correlated with the level of NQO-1. (F) EZH2 mRNA level is negatively correlated with the level of HO-1.

### 3.3. EZH2 is correlated with Nrf2 expression in NSCLC tissues

As we have showed that EZH2 controlled lung cancer growth and predicted patients' survival. We next wanted to know the

underlying mechanism by which EZH2 regulates lung cancer growth. Nrf2 is a transcription factor that was reported to regulate lung cancer proliferation and drug-resistance [11,15,21,22]. Accumulating data have shown the high expression of Nrf2 in

the tissues of human NSCLC as described in the introduction. However, how Nrf2 is regulated at the transcription level remains elusive. A previous report showed that when cancer cells were treated with proteasome inhibitor MG132, EZH2 was down-regulated, which was coupled with the up-regulation of Nrf1 and Nrf2 [23]. Therefore, we hypothesized that EZH2 may regulate Nrf2 expression in lung cancer cells. First, we explored the expression level of Nrf2 and its downstream genes (*NQO-1* and *HO-1*) in lung cancer tissues and adjacent normal tissues. We found that Nrf2 was overexpressed in human NSCLC tissues (Figs. 3A and 4D). In addition, *NQO-1* and *HO-1*, two genes transcriptionally controlled by Nrf2 and participate significantly in NSCLC growth and drug-resistance, were also up-regulated in NSCLC tissues (Fig. 3B and C). Next, we performed linear regression analysis to analyze the correlation between *EZH2* mRNA level and *Nrf2*, *NQO-1* and *HO-1* mRNA levels. The results showed that *EZH2* mRNA level was negatively but significantly correlated with the mRNA levels of *Nrf2*, *NQO-1* and *HO-1* (Fig. 3D–F). Interestingly, we found that EZH2 can bind to the promoter region of *Nrf2* gene in lung cancer tissues and cell lines (Fig. 4A, Supplementary Fig. 4), but not to the promoter region of *NQO-1* or *HO-1* gene (Supplementary Fig. 5). As EZH2 is an H3K27me3 methyltransferase, we also investigated the enrichment of H3K27me3 at the promoter region of *Nrf2* gene. The results showed that the H3K27me3 at *Nrf2* promoter was trimethylated (Fig. 4B). Interestingly, EZH2 level and the methylation level of H3K27me3 at *Nrf2* promoter was significantly down-regulated in NSCLC tissues compared with adjacent normal tissues (Fig. 4C). In addition, we also found the global decrease in H3K27me3 level in NSCLC tissues compared to adjacent normal tissues (Fig. 4D). Because H3K27me3 is correlated with transcription repression, we wanted to know whether low methylation of H3K27me3 enrichment at *Nrf2* promoter is correlated with high *Nrf2* expression. Our linear regression analysis indicated that H3K27me3 enrichment at *Nrf2* promoter was negatively but

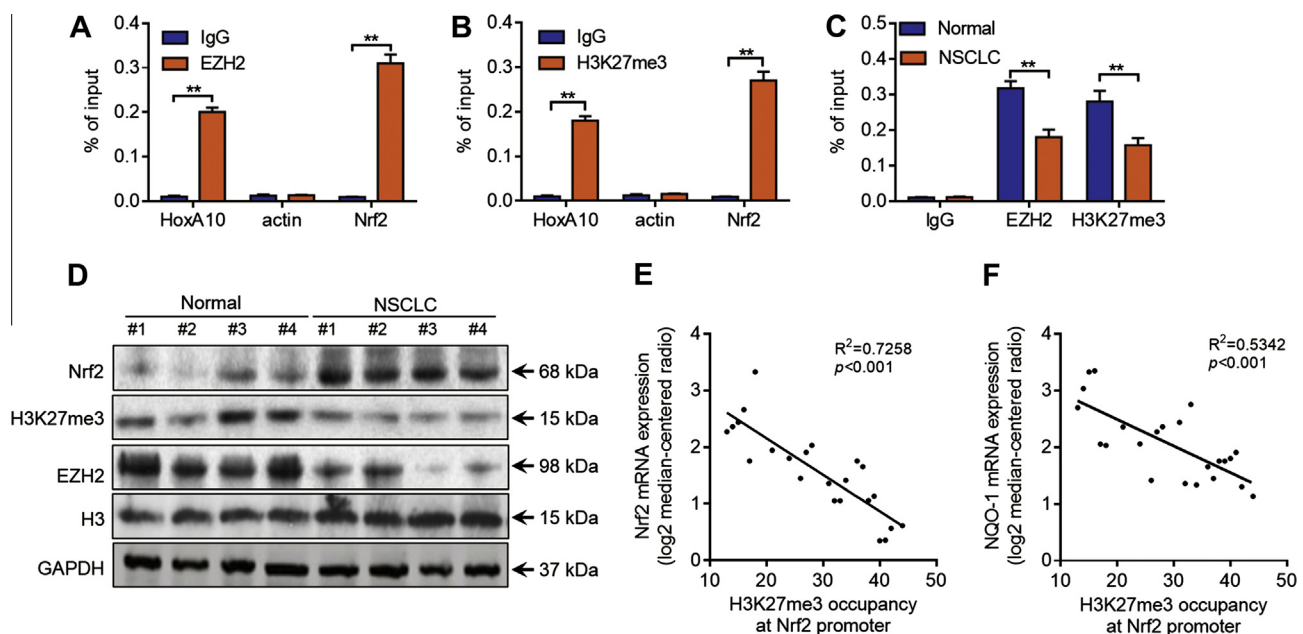
significantly correlated with the expression level of *Nrf2* and its downstream genes (Fig. 4E, F and data not shown).

#### 3.4. EZH2 regulates *Nrf2* expression through trimethylation of H3K27

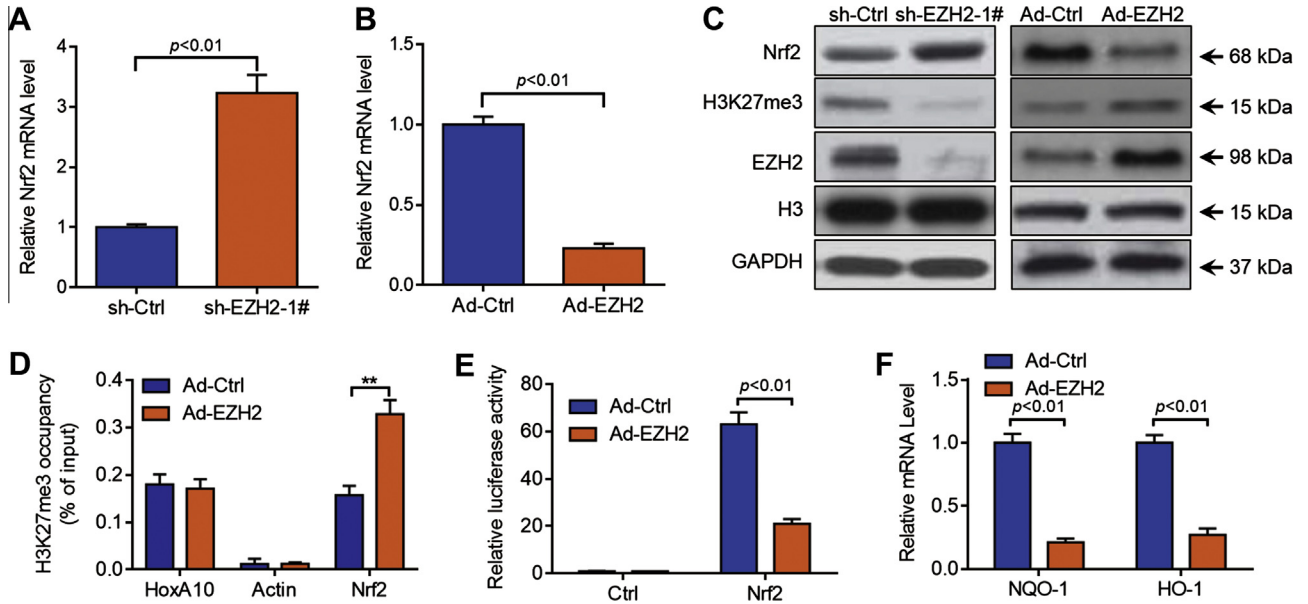
To further demonstrate the effect of EZH2 on Nrf2 and its downstream genes, we knocked down or overexpressed EZH2 in A549 lung cancer cells. We found that EZH2 knockdown significantly up-regulated the mRNA level of *Nrf2* (Fig. 5A), while EZH2 overexpression down-regulated the mRNA level of *Nrf2* (Fig. 5B). In line with this finding, EZH2 knockdown decreased H3K27me3 level and up-regulated Nrf2 protein level, whereas EZH2 overexpression increased H3K27me3 level and down-regulated Nrf2 protein level (Fig. 5C). In addition, EZH2 overexpression enhanced H3K27me3 enrichment at the promoter of *Nrf2* (Fig. 5D), indicating that EZH2 may inhibit *Nrf2* promoter activity. Indeed, our luciferase assay showed that EZH2 overexpression inhibited the activity of *Nrf2* promoter (Fig. 5E). Finally, we analyzed the mRNA level of *Nrf2* downstream genes in A549 cells with EZH2 overexpression. The results showed that EZH2 overexpression significantly down-regulated the mRNA levels of *NQO-1* and *HO-1* (Fig. 5F). Those findings provide direct evidence that EZH2 inhibits Nrf2 expression by regulating H3K27me3. As previous report has showed that EZH2 expression was correlated with Nrf1 level when cancer cells were treated with proteasome inhibitor MG132, we also assessed the effect of EZH2 on *Nrf1* expression. However, our data showed that neither EZH2 overexpression nor EZH2 knockdown could alter the mRNA level of *Nrf1* in A549 cells (Supplementary Fig. 6).

#### 3.5. *Nrf2* is essential for the function of EZH2 in A549 lung cancer cell proliferation

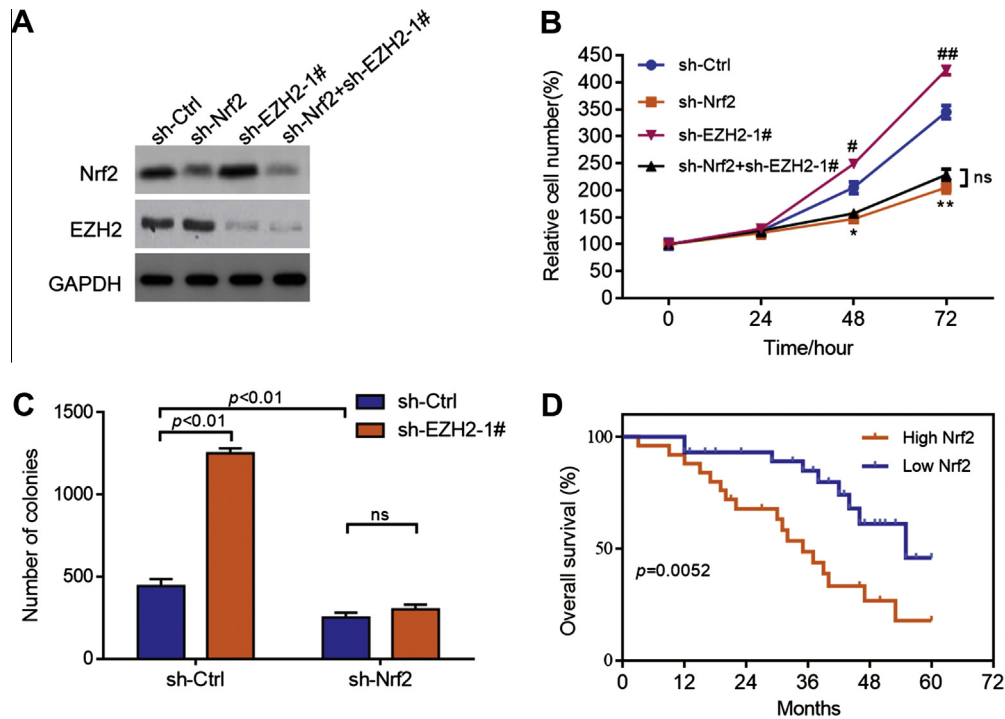
Our data have demonstrated that EZH2 inhibited Nrf2 expression through regulating H3K27me3 methylation at *Nrf2* promoter.



**Fig. 4.** H3K27me3 is involved in EZH2 effects on Nrf2 expression. (A, B) NSCLC tissues were subjected to ChIP analysis with anti-EZH2, anti-H3K27me3 or control anti-IgG antibodies. The values were expressed as percentage of input. HoxA10 serves as positive control and Actin as negative control. (A) EZH2 can bind to the promoter region of *Nrf2* gene.  $N = 5$  in each group. (B) H3K27me3 is enriched at the promoter region of *Nrf2* gene.  $N = 5$  in each group. (C) EZH2 and H3K27me3 enrichment on *Nrf2* promoter region is decreased in NSCLC tissues compared to that in normal tissues. NSCLC tissues and normal tissues were subjected to ChIP analysis with anti-EZH2 or anti-H3K27me3 antibody respectively. Anti-IgG antibody served as a control.  $N = 8$  in each group. (D) Global H3K27me3 level is decreased in NSCLC tissues compared to that in normal tissues. Protein was extracted from NSCLC and normal tissues and subjected to Western blot analysis with indicated antibodies. (E, F) Linear regression analysis was performed to analyze the correlation between H3K27me3 enrichment at *Nrf2* promoter and *Nrf2* (E) or *NQO-1* (F) mRNA level.



**Fig. 5.** EZH2 inhibits Nrf2 expression by targeting H3K27me3. A549 cells were infected with retro-sh-Ctrl, retro-sh-EZH2-1#, Ad-Ctrl or Ad-EZH2 for 48 h, then RNA and protein were extracted and subjected to q-PCR analysis (A, B, F) or Western blot analysis (C) respectively. (A) EZH2 knockdown increases *Nrf2* mRNA level. (B) EZH2 overexpression decreases *Nrf2* mRNA level. (C) EZH2 knockdown decreases H3K27me3 level and increases Nrf2 protein level, while EZH2 overexpression increases H3K27me3 level and decreases Nrf2 protein level. (D) EZH2 overexpression increases H3K27me3 enrichment at *Nrf2* promoter. A549 cells were infected with Ad-Ctrl or Ad-EZH2 for 48 h. Then the samples were subjected to ChIP analysis with indicated antibodies. *HoxA10* serves as a positive control and Actin as a negative control. (E) Luciferase assay showing that EZH2 overexpression decreases *Nrf2* promoter activity. (F) EZH2 overexpression decreases *NQO-1* and *HO-1* mRNA level. \*\* $P < 0.01$ .



**Fig. 6.** Nrf2 is essential for EZH2 effects on lung cancer cell growth. (A) Nrf2 and EZH2 knockdown in A549 cells. (B) Nrf2-knockdown blocks EZH2's effects on cell proliferation. A549 cells stably expressing sh-Ctrl or sh-Nrf2 were infected with retrovirus carrying sh-Ctrl or sh-EZH2. The relative cell number was evaluated at the indicated time points. \* $P < 0.05$ ; \*\* $P < 0.01$  indicate sh-Ctrl versus sh-EZH2; # $P < 0.05$ ; ## $P < 0.01$  indicate sh-Ctrl versus sh-sh-Nrf2. (C) Nrf2-knockdown blocks EZH2's effects on colony formation. A549 cells stably expressing sh-Ctrl or sh-Nrf2 were infected with retrovirus carrying sh-Ctrl or sh-EZH2. Then the cells were subjected to soft sugar colony formation assay. (D) High Nrf2 level predicts poor survival. Kaplan–Meier curve comparing time to survival between NSCLCs with the low versus high *Nrf2* expression was determined using the q-PCR results.

Another question remains unknown is whether Nrf2 is essential for the role of EZH2 in lung cancer cells. We generated A549 cells with stably Nrf2 knockdown (Fig. 6A), then we infected those cells with

retrovirus carrying Ctrl shRNA, and EZH2 shRNA. We found that EZH2 knockdown was unable to affect either A549 cell proliferation (Fig. 6B) or colony formation capacity (Fig. 6C) when Nrf2

was knocked down, whereas modulation of EZH2 could still inhibit A549 cell proliferation and colony formation when Nrf2 shRNA was replaced by a control shRNA (Fig. 6B and C). Finally, we showed that high Nrf2 level predicted poor survival (Fig. 6D). Those findings demonstrate that Nrf2 is critically essential for the role of EZH2 in lung cancer cells.

#### 4. Discussion

EZH2 was first reported to participate in the progression of prostate cancer ten years ago [6]. Ectopic expression of EZH2 in prostate cells induces transcriptional repression of a specific cohort of genes. Gene silencing mediated by EZH2 requires the SET domain and is attenuated by inhibiting histone deacetylase activity. Amounts of both EZH2 messenger RNA and EZH2 protein are increased in metastatic prostate cancer; in addition, clinically localized prostate cancers that express higher concentrations of EZH2 show a poorer prognosis [6]. EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells [7]. Mechanistically, EZH2 mediates transcriptional silencing of the tumor suppressor gene E-cadherin by trimethylation of H3K27 [24]. EZH2 is essential for the proliferation of both transformed and non-transformed human cells [25]. Clinical evidence identified EZH2 as a novel and independent prognostic marker in endometrial cancer, and validate previous findings on prostate and breast cancer [26]. Interestingly, a recent report showed that EZH2 serves as a tumor suppressor in myelodysplastic syndromes, which was evidenced by *EZH2* deletions, missense and frameshift mutations [27]. Here we showed the down-regulation of EZH2 in human NSCLC tissues. We further found that EZH2 serves as a tumor suppressor in lung cancers.

Nrf2 has long been considered to be a factor against environmental oxidative stress. However, recently evidence have showed that Nrf2 can promote cancer cell proliferation and regulate drug resistance. Accumulating data have strongly demonstrated the oncogenic role of Nrf2 in human NSCLC. Loss of Keap1 or decreased Keap1 activity in cancer cells induces greater nuclear accumulation of Nrf2, causing enhanced transcriptional induction of antioxidants, xenobiotic metabolism enzymes, and drug efflux pumps [14–16]. Cancer related mutations in *Nrf2* impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy [28]. Nrf2 enhances cell proliferation and resistance to anticancer drugs in human lung cancer [11]. In addition, a recent report showed that gain of Nrf2 function in NSCLCs confers radio-resistance [17]. Our recent work identified acetylation affects Nrf2 function in human lung cancer. We showed that hMOF, the histone acetyltransferase required for histone H4 lysine 16 acetylation, can bind to Nrf2 and acetylates Nrf2. hMOF-dependent Nrf2 acetylation maintains its retention in the nucleus and mediates the transactivity of Nrf2, subsequently regulates lung cancer growth and drug-resistance [18]. However, the mechanism by which Nrf2 is regulated at the transcription level remains unknown. Herein we showed that Nrf2 is inhibited by EZH2. The expression of Nrf2 and its downstream genes are correlated with EZH2 expression and H3K27me3 level in human NSCLC samples. Further, EZH2 could bind to *Nrf2* promoter and regulated the trimethylation of H3K27, which repressed *Nrf2* transcription. In lung cancer tissues, EZH2 down-regulation or mutation leads to the reduction of H3K27me3 at the promoter of *Nrf2*, resulting in up-regulation of *Nrf2* transcription. However, translated Nrf2 proteins might be unable to degenerate timely, as previous report has showed mutations in *KEAP1* in approximately 20% of patients and 50% of cell lines in NSCLC, which leads to Nrf2 phosphorylation/acetylation and nuclear translocation [14,18]. Together with our previous

report, we identified the underlying mechanism by which Nrf2 is regulated at transcriptional and post-translational levels. Finally, Nrf2 was critically essential for the role of EZH2 in human lung cancer cells, and high Nrf2 level predicts poor survival.

In summary, low *EZH2* expression predicts poor survival in human NSCLC. Our in vitro and in vivo evidence demonstrate that EZH2 inhibits lung cancer cell growth. Mechanistically, EZH2 binds to the promoter of *Nrf2* and represses Nrf2 expression by regulating H3K27me3. Finally, we show that Nrf2 is essential for the role of EZH2 in lung cancer cells.

#### 5. Conflict of interest

None.

#### Acknowledgements

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.febslet.2014.05.057>.

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