SETDB1 accelerates tumourigenesis by regulating the WNT signalling pathway

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Abstract

We investigated the oncogenic role of SETDB1, focusing on non-small cell lung cancer (NSCLC), which has high expression of this protein. A total of 387 lung cancer cases were examined by immunohistochemistry; 72% of NSCLC samples were positive for SETDB1 staining, compared to 46% samples of normal bronchial epithelium (106 cases) (p < 0.0001). The percentage of positive cells and the intensity of staining increased significantly with increased grade of disease. Forced expression of SETDB1 in NSCLC cell lines enhanced their clonogenic growth in vitro and markedly increased tumour size in a murine xenograft model, while silencing (shRNA) SETDB1 in NSCLC cells slowed their proliferation. SETDB1 positively stimulated activity of the WNT–β-catenin pathway and diminished P53 expression, resulting in enhanced NSCLC growth in vitro and in vivo. Our finding suggests that therapeutic targeting of SETDB1 may benefit patients whose tumours express high levels of SETDB1.

Keywords: SETDB1; WNT; tumourigenesis; lung

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No conflicts of interest were declared.

Introduction

Lung cancer is the most common cancer worldwide, accounting for 1.3 million deaths annually. Non-small cell lung cancer (NSCLC) represents about 80% of lung cancers [1]; the patients are often diagnosed at advanced stages, when surgical cure is no longer possible [2]. Several oncogenic drivers have been identified for this cancer. However, currently only two categories of targeted therapies are available: one is gefitinib/erlotinib, tyrosine kinase inhibitors targeting the mutated epidermal growth factor receptor (EGFR) occurring especially in Asian patients (30–50%) [3,4]; the other is crizotinib, a kinase inhibitor targeting the echinoderm microtubule-associated protein-like 4 anaplastic lymphoma kinase (EML4–ALK) fusion protein (approximately 4–7% of patients) [5,6]. A large majority of NSCLC patients receive conventional chemotherapy, associated with toxicity and often only a marginal survival benefit. Searching for additional aberrant pathways in NSCLC is needed to identify novel 'druggable' targets, leading to the development of new targeted therapeutic strategies.

Many biological processes, such as gene transcription, genome stability and recombination/DNA repair, are controlled by chromatin structures [7]. The primary architecture of chromatin is organized by histones, a class of small nuclear proteins which can be modified by acetylation, ubiquitination, phosphorylation or methylation [7,8]. These modifications, also referred to as the 'histone code', coordinate and help control genomic transcription and play a crucial role in maintaining genomic stability [9,10]. Overall, acetylation of histones usually marks a transcriptional activating region, and the process is tightly controlled by...
histone acetyltransferases (HATs) and histone deacetylases (HDACs) [11]. In contrast, methylation of histones results in a much more complex and diverse regulatory pattern. Generally, methylation of histone H3 lysine 4 (H3K4), lysine 36 (H3K36) and lysine 79 (H3K79) is associated with gene activation, whereas methylation of histones H3K9, H3K27 and H4K20 usually leads to gene repression [12]. The process of histone methylation is, in part, dynamically controlled by a variety of SET domain-containing methyltransferases and demethylases [13].

SETDB1 is a histone H3 lysine 9 methyltransferase, which was initially identified as a binding partner of KRAB-associated protein-1 (KAP1) [14]. It transfers the methyl group(s) to histone H3K9, helping to control heterochromatin formation and chromatin organization [15]. SETDB1 is also required for efficient endogenous proviral silencing during early embryogenesis, which is indispensable for mammalian embryonic development [16].

Materials and methods

Patient samples
Sixty primary NSCLC samples and their paired adjacent normal tissue (for SETDB1 mRNA expression analysis by RT–PCR) were obtained from Shanghai Chest Hospital, with consent obtained from patients [17,18]. The lung cancer tissue array used in the immunohistochemical (IHC) assay was described previously (IRB Protocol No.02-07-01-I-13; UCLA Institutional Medical Review Board) [19,20].

Immunohistochemical assays
Polyclonal rabbit anti-SETDB1 antibody obtained from Sigma-Aldrich (HPA018142) was used for the IHC experiments after specificity testing (see supplementary material, Figure S1). Detailed IHC assays are described in Supplementary materials and methods (see supplementary material).

Cell lines and cell culture
NSCLC cell lines PC14, A549, HCC2279, H1299, H23 and HCC1975 were grown in RPMI 1640 medium plus 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO₂ at 37°C.

Generation of stably-transfected cell line
The entire coding region of SETDB1 was amplified from pcMV2–SETDB1, which was a generous gift from Dr Frank J Rauscher III. The lentiviral SETDB1 over-expression and shRNA vectors were generated as described in Supplementary materials and methods (see supplementary material). The target sequences of these shRNA are listed in Table S1 (see supplementary material). Stably transfected cell lines were obtained by selection with 2–5 μg/ml puromycin.

RNA and protein extraction
Total RNA was extracted from either cell lines or xenograft tissues using a QIAamp RNA kit (Qiagen). Cells were lysed with M-PER Mammalian Protein Extraction Reagent (Thermo Scientific), containing protease inhibitor cocktail (Roche Life Science). Protein quantification was determined using the BCA Protein Assay Kit (Thermo Scientific).

Microarray analysis
Microarray analyses were performed with H1299 cells expressing either GFP or SETDB1. Triplicate experimental and control samples were used for the analysis. The array hybridization was performed using the Human HT-12 v. 4 chip Expression BeadChip (Illumina). Pathway analysis was accomplished using the KEGG database. Real-time PCR (RT–PCR) was performed to validate the selected genes.

Western blot analysis
Western blot analysis was performed with the primary antibodies against SETDB1 (Sigma-Aldrich and Proteinitech group), α-tubulin (Sigma-Aldrich), β-actin (Sigma-Aldrich), β-catenin (Cell Signaling Technology), Histone H3 (Cell Signaling Technology), Cyclin B1 (Cell Signaling Technology), Cyclin D1 (Santa Cruz Biotechnology), c-MYC (Santa Cruz Biotechnology) and H3K9me1/me2/me3 (Upstate Biotechnology).

Colony formation assays
Colony formation assays were performed in 1 ml cultures in 12-well flat-bottomed plates. The base layer (0.5 ml) contained 0.25 ml 1% low-melting agarose and 0.25 ml 2× RPMI with 20% FBS. After solidified, cells were added and mixed into the upper layer (0.5 ml), containing 0.25 ml 0.7% low-melting agarose and 0.25 ml 2× RPMI with 20% FBS. The plates were incubated for 21–35 days at 37°C in a fully humidified atmosphere of 5% CO₂ in the air. Colony numbers were calculated after staining with crystal violet.

Luciferase reporter assay
The Dual Luciferase Reporter Assay system (Promega) was used according to the manufacturer’s instructions. GFP control or SETDB1 stably transfected cells were seeded in 0.5 ml medium in 12-well tissue-culture plates. Cells were transfected with 1 μg of either TOP or FOP flash and 0.5 ng pRL-TK vector; 72h after transfection, the levels of WNT pathway activity were measured by luciferase activity.

ChIP assay
ChIP assays were performed according to the protocol described previously (http://www.lbl.gov/LBL-Pro grams/lifesciences/BissellLab/labprotocols/chip.htm). Briefly, 7 million H1299 cells over-expressing SETDB1
were crosslinked with 1% formaldehyde for 10 min. Lysates were sonicated on ice to shear DNA to lengths of 500–800 bp. Chromatin was immunoprecipitated overnight at 4°C with either anti-SETDB1 antibody (Proteintech group) or normal rabbit IgG (Santa Cruz Biotechnology). ChIP-enriched DNA was quantified by quantitative PCR and the PCR product was further examined by agarose gel electrophoresis and ethidium bromide staining.

Animal studies
Age-matched nude mice (aged 5–6 weeks) were used for the in vivo xenograft experiments. A total of 3 million H1299 cells, stably expressing either SETDB1 or GFP (control), were suspended in a 1:1 mixture of FBS and Matrigel (BD Labware) and injected subcutaneously into both flanks of nude mice.

Results
SETDB1 is recurrently amplified and highly expressed in NSCLC patients
Gene copy number alterations were examined by 250k SNP-Chip analysis in nine NSCLC cell lines [17]. Gain or amplification (Amp) of chromosome 1q21 was identified as a common alteration among these cell lines. SETDB1 is one of the possible target genes located in this region, which prompted further investigations. Tumours in the TCGA (6547 tumour samples) and Tumorscape (3131 tumour samples) databases were examined for the SETDB1 copy number changes. SETDB1 was significantly amplified in ~20% of NSCLC samples in the TCGA compilation (with a focal frequency of 0.2159; Q value 5.08 E-21) and in the Tumorscape dataset (with a focal frequency of 0.2074; Q value 3.45 E-31). The results are summarized in Figure 1A, B (see also supplementary material, Figure S2).

Next, we explored whether either copy number gain or amplification in the genomic DNA translated into elevated SETDB1 expression. Microarray expression data were examined for each patient who had also been analysed for SETDB1 copy number, using the TCGA lung adenocarcinoma dataset. Compared to the samples grouped as either heterozygous loss (Hetloss) and diploid cohorts, SETDB1 mRNA levels were significantly elevated in those grouped as either gain (Gain) or amplified (Amp) in copy number of the SETDB1 cohorts (Figure 1C), indicating that the copy number gain or amplification of SETDB1 loci resulted in elevated SETDB1 transcripts in lung cancer samples. Indeed, the elevation of SETDB1 transcripts were also noted in eight independent expression microarray datasets of lung cancer, which were done by different research groups; expression data were collected from the GEO or Expression Atlas (EMBL-EBI) databases (Figure 1D). Furthermore, patients with elevated SETDB1 expression displayed a worse outcome compared to those with a lower SETDB1 expression (GSE14814) (Figure 1E).

To confirm our in silico observations, we independently interrogated our own NSCLC patient sample collection (NSCLC versus normal lung matched pairs). Among 60 matched samples, 23 NSCLC tumours had up-regulation of SETDB1 mRNA by >2-fold (Figure 1F). In addition, elevated expression of SETDB1 was noted in the tumours which were either grade 3 or 4 tumours (Figure 1F).

We then examined SETDB1 protein expression in 387 lung cancer samples and 106 normal lung samples by tissue microarray (TMA). Details of the patient information are listed in Figure 2A. Representative examples of tumour sections with either strong or weak nuclear staining of SETDB1 are shown in Figure 2B. A mean 71.5% of NSCLC cells were positive for SETDB1, compared to 45.5% in normal bronchial epithelium cells (p <0.0001); the mean intensity value of SETDB1 staining was significantly higher in tumours than in the normal control samples (1.06 versus 0.74; Figure 2C). Both the percentage positive cells and intensity of staining for SETDB1 were greater in NSCLC cells than the non-malignant bronchial epithelium control group ("bronch"); a significant association was consistently noted between SETDB1 expression and tumour grade (Figure 2D, E).

SETDB1 promotes anchorage-independent growth in vitro and tumour growth in vivo
To test the functional importance of our findings, we silenced SETDB1 expression in several NSCLC cell lines, using three shRNAs targeting different coding regions of the gene. Silencing SETDB1 slowed proliferation in the cell lines A549, PC14 and HCC2279, whereas the changes were not significant in H1299 and H23 cells (Figure 3A). In contrast, SETDB1 silencing reduced the anchorage-independent clonogenic growth capacity of all five of these NSCLC cell lines when examined by soft agar clonogenic assays (Figure 3B). Reduction of clonogenic growth was especially prominent in TP53-deficient cell lines, with decreased clonal growth of 86%, 71% and 60% in SETDB1-silenced PC14, H23 and H1299 cells, respectively (detailed mutational information of cell lines is described in Table S2; see supplementary material). On the other hand, significantly elevated clonogenic growth was observed in cell lines with forced expression of SETDB1 (Figure 3C). This was especially dramatic (five-fold) in the H1299 cells having deletion of TP53 (Figure 3C). Consistent with the characterized role of SETDB1 as a histone H3K9 methyltransferase, elevated H3K9 di- and tri-methylation was noted in both H1299 and A549 cell lines when SETDB1 was over-expressed (Figure 3D).

To analyse the effect of SETDB1 on cell growth in vivo, xenografts were established by subcutaneous injection of H1299 cells having forced expression of either SETDB1 or GFP into an immunodeficient murine...
Figure 1. Elevated expression of SETDB1 in NSCLC patients. (A) Copy number alterations of SETDB1 across a variety of cancer types (TCGA and Tumorscape database). SETDB1 is located at the peak region of the amplicon in lung cancer in both lung cancer sample collections; significance was defined as Q value (calculation was performed on the database servers). (B) Heat map schematic illustration of copy number amplification of SETDB1 in Tumorscape lung cancer samples, which harbour an 1q21 amplification; the position of SETDB1 is indicated. (C) Gain of SETDB1 DNA copy number correlated with increased mRNA expression in the NSCLC samples. (D) Elevated expression of SETDB1 was noted in eight different lung cancer patient cohorts; data were retrieved from the GEO and EBI Gene Expression Atlas database (*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001). (E) Kaplan–Meier plots of overall survival: comparison of cases with highest (20 patients) versus lowest (20 patients) expression of SETDB1 in NSCLC patients (GSE14814); p value was calculated by log-rank test. (F) mRNA levels of SETDB1 (examined by real-time PCR) in a set of 60 paired samples of NSCLC; (inner image) elevated expression of SETDB1 correlates with increase of tumour grade.

model (Figure 3E). Tumour sizes and weights were significantly increased in mice injected with forced expression of SETDB1 H1299 cells compared with GFP control cells (p = 0.0003; Figure 3F). Total RNA and protein was extracted from the tumours and the over-expressions of SETDB1 in these tumours were further confirmed by both RT–PCR and western blot (Figure 3G).

We also explored the possible interaction of P53 and SETDB1. Interestingly, forced silencing of TP53 in SETDB1 over-expressing A549 cells (TP53 wild-type) resulted in a remarkable increase of their clonogenic
growth in soft agar (Figure 4A) as well as the in vivo xenografts, although the latter was not statistically significant (Figure 4B, C; p = 0.18). This suggests that a functional TP53 may antagonize the SETDB1 oncogenic effect. We made use of the isogenetic TP53−/− vs TP53+/+ HCT116 model to examine further the SETDB1 expression. Both the mRNA and protein levels of SETDB1 were increased in the TP53−/− compared to the TP53+/+ HCT116 cells. On the other hand, forced expression of SETDB1 significantly reduced the protein level of TP53. Likewise, silencing SETDB1 in three NSCLC lines increased their protein levels of P53 (Figure 4E). Taken together, SETDB1 and TP53 appear to modulate expression of each other.

SETDB1 regulates the WNT pathway

To investigate the molecular mechanism underlying the SETDB1 contribution to enhanced growth of lung cancer, we utilized cDNA microarray to profile gene expression changes in H1299 cells (Figure 5A). A total of 711 genes were down-regulated and 522 genes were up-regulated after forced expression of SETDB1. Pathway analysis showed that cancer-related genes were significantly altered in SETDB1-over-expressing cells (p < 0.0005). In addition, genes related to signalling by Toll-like receptors, insulin, WNT, MAPK, focal adhesion and the JAK–STAT pathway were also significantly enriched (Figure 5B). The WNT pathway was selected for further study because both the SETDB1 and WNT pathways are critical to the maintenance of self-renewal of stem cells [21–23]. Four WNT-relevant genes identified by RNA array were validated by RT–PCR: APOE (four-fold down-regulation); IGFBP4 (three-fold up-regulation); FZD1 (1.7-fold up-regulation); and LRP8 (1.7-fold up-regulation) (Figure 5C). We examined whether SETDB1 regulated these genes by binding directly to their promoter regions. Each promoter was divided into 10–14 sections

Figure 2. SETDB1 protein expression is elevated in NSCLC samples. (A) Subtypes of lung cancer samples examined by immunohistochemistry (IHC). (B) Representative examples of SETDB1 staining in NSCLC tumour sections: (upper panel) strong SETDB1 staining; (lower panel) weak SETDB1 staining. (C) Total of 387 NSCLC cases was analysed by IHC; SETDB1-positive staining cells (upper row) and integrated intensities (lower row) in NSCLC samples compared to normal bronchial epithelium. (D) SETDB1-positive staining cells (%; upper row) and intensity of staining (lower row) correlates with tumour grade. (E) SETDB1 expression in different subtypes of lung cancer; data are presented as percentage positivity (upper row), and intensity of staining (lower row) is higher in each different subtype of cancer compared to normal growth in soft agar (Figure 4A) as well as the in vivo xenografts, although the latter was not statistically significant (Figure 4B, C; p = 0.18). This suggests that a functional TP53 may antagonize the SETDB1 oncogenic effect. We made use of the isogenetic TP53−/− vs TP53+/+ HCT116 model to examine further the SETDB1 expression. Both the mRNA and protein levels of SETDB1 were increased in the TP53−/− compared to the TP53+/+ HCT116 cells. On the other hand, forced expression of SETDB1 significantly reduced the protein level of TP53. Likewise, silencing SETDB1 in three NSCLC lines increased their protein levels of P53 (Figure 4E). Taken together, SETDB1 and TP53 appear to modulate expression of each other.

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Figure 3. Aberrant expression of SETDB1 in NSCLC cells affects their proliferation in liquid culture, clonogenic growth in semi-soft cultures and tumour size in nude mice. (A) Effect of stably silencing SETDB1 on NSCLC cell growth in liquid culture; MTT assay was performed in 96-well plates; mean ± SD of six wells. (B) Effect of silencing of SETDB1 on colony formation of NSCLC cells; cells were seeded into soft agar in triplicate, and colonies were counted after 21–28 days of culture; mean ± SD (three wells) are expressed as percentage variation relative to scrambled shRNA-infected cells (control). (C) Effect of forced expression of SETDB1 on colony formation of PC14 and H1299 NSCLC cells; cells were seeded into soft agar in triplicate dishes and colonies were counted on day 28 of culture; values are expressed as fold variation relative to GFP over-expressing cells (control); results are mean ± SD of three experiments; (right panel) representative images of colonies of H1299 over-expressing SETDB1. (D) Histone H3K9 methylation status of NSCLC cells with stable over-expression of SETDB1 (H1299 and A549) was examined by western blot, using antibodies specific for H3K9me1, H3K9me2 and H3K9me3 (mono-, di- and tri- methylation of H3K9, respectively); expression levels of histone H3 protein were used as an internal control. (E) SETDB1 stimulates NSCLC tumour growth in mice: photograph of tumours dissected from nude mice, which were injected with H1299 cells over-expressing either GFP (upper row) or SETDB1 (lower row); tumour cells ($3 \times 10^6$) were suspended in a 1:1 mixture of FBS and Matrigel (BD Labware) and injected into both flanks of 5 week-old nude mice; the tumours were removed on day 21 from initiation of the experiment. (F) Weights of tumours shown in (E); the bars show differences in average weight of tumours in the two groups (mean ± SD, $n=8$); difference of mean weights between control (GFP) and SETDB1 over-expressing tumours was statistically significant ($p=0.0003$). (G) Expression level of SETDB1 in the tumours with forced expression of either GFP or SETDB1; proteins and mRNA were isolated from xenografts; tumour mRNA values were measured by RT–PCR and normalized with β-actin; values are expressed as fold variation of SETDB1 over-expressing tumours relative to GFP control tumours; SETDB1 protein was measured by western blot (inner image).  

(∼200 bp) and ChIP-PCR was performed to determine the SETDB1 binding regions (see supplementary material, Table S2). Three SETDB1 enrichment sites were identified in the promoter region of APOE at −850, −1600 and −2700 bp upstream from the start codon ATG, as well as 500 bp downstream of the start codon (Figure 5D, upper and left panels). In addition, three regions of enrichment occurred in the promoter of IGFBP4 (gene up-regulated by SETDB1; Figure 5D, lower and right panels); but no enrichment was detected in the promoter region of the other two SETDB1-up-regulated genes, FZD1 and LRP8 (data not
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Figure 4. Inverse correlation between the protein levels of P53 and SETDB1. (A) Silencing TP53 enhanced the colony formation of A549 cells (wild-type P53) having forced expression of SETDB1. (B) A549 NSCLC cells were either stably over-expressing SETDB1 (upper row) or stably over-expressing SETDB1 and silencing TP53 (lower row): these two cohorts of cells (5 × 10^6) were injected into the opposite flanks of nude mice; tumours were photographed (B) and weighed (C) on day 35 after initiation of the study. (D) SETDB1 expression levels in the HCT116 TP53+/− and TP53−/− HCT116 cells: (left panel) SETDB1 mRNA expression; (right panel) western blot of the same cells. (E) Protein levels (western blot) of P53 and SETDB1 in NSCLC cells (PC14, HCC1975, H23) with either stable over-expression or silencing of SETDB1 shown), suggesting that up-regulation of these genes may not be directly controlled by SETDB1. Consistent with SETDB1 directly binding to the promoter region of APOE and IGFBP4, silencing of SETDB1 significantly down-regulated IGFBP4 and up-regulated APOE mRNA, but did not affect the mRNA levels of β-catenin (Figure 5E).

Since APOE, FZD1, LRP8 and IGFBP4 all participated in WNT–β-catenin signalling by inhibiting the degradation of β-catenin protein [24–28], we speculated that alteration of these genes may lead to accumulation of β-catenin protein and activation of the WNT pathway. As anticipated, western blot demonstrated a prominent elevation of β-catenin protein in SETDB1-over-expressing cells and a decreased level of β-catenin protein when silencing SETDB1 in NSCLC (Figure 6A). As SETDB1 caused no significant change of β-catenin mRNA in either SETDB1-over-expressed or -silenced cells (Figure 5C, E), the pathway of accumulation of β-catenin is probably post-transcriptional.

Activation of the WNT pathway is required for SETDB1-induced tumourigenesis

To prove further that SETDB1 increased the activity of the WNT pathway, the TOP/FOP reporter system was employed to measure the activity of the WNT pathway [29]. Over-expression of SETDB1 in H1299 cells significantly increased the value of TOP luciferase activity (Figure 6B). Consistently, silencing of either FZD1, LRP8 or IGFBP4 significantly reduced TOP luciferase activity in H1299 cells (Figure 6C). Since the hallmark of activation of WNT signalling pathway is nuclear localization of β-catenin [30–32], the total proteins of transfected H1299 cells were separated into cytoplasmic and nuclear fractions to examine the nuclear β-catenin accumulation. Lysates were collected, blotted and probed with β-catenin antibody. Examination of the cytoplasmic protein α-tubulin and the nuclear protein Histone H3 (compartment-specific controls) confirmed good nuclear–cytoplasmic protein separation. A significant increase of β-catenin protein migrated into the nucleus of SETDB1 over-expressing H1299 cells (Figure 6D), consistent with activation of the WNT pathway in SETDB1 over-expressing cells. When examining the downstream pathway of WNT, the elevated protein levels of β-catenin stimulated by SETDB1 were associated with increased levels of c-MYC and Cyclin D1 (Figure 6E).

Rescue experiments were carried out to test the concept that activation of the WNT pathway is required for transformation induced by SETDB1. Depletion of β-catenin abolished the colony-forming ability of SETDB1-over-expressing H1299 cells (Figure 6F). Furthermore, silencing of either FZD1, LRP8 or IGFBP4 concomitantly with forced expression of SETDB1 also suppressed the enhanced clonogenic and liquid culture growth of SETDB1-over-expressing cells (Figure 6G; see also supplementary material, Figure S3). Taken together, these results highlight the critical role of the β-catenin pathway in SETDB1-mediated transformation.

Discussion

SETDB1 is within the 1q21 amplicon, a region recurrently amplified in a variety of solid tumours, including lung, breast and ovarian cancers as well as

Figure 4. Inverse correlation between the protein levels of P53 and SETDB1. (A) Silencing TP53 enhanced the colony formation of A549 cells (wild-type P53) having forced expression of SETDB1. (B) A549 NSCLC cells were either stably over-expressing SETDB1 (upper row) or stably over-expressing SETDB1 and silencing TP53 (lower row): these two cohorts of cells (5 × 10^6) were injected into the opposite flanks of nude mice; tumours were photographed (B) and weighed (C) on day 35 after initiation of the study. (D) SETDB1 expression levels in the HCT116 TP53+/− and TP53−/− HCT116 cells: (left panel) SETDB1 mRNA expression; (right panel) western blot of the same cells. (E) Protein levels (western blot) of P53 and SETDB1 in NSCLC cells (PC14, HCC1975, H23) with either stable over-expression or silencing of SETDB1
Figure 5. Altered gene expression in NSCLC cells with forced expression of SETDB1. (A) RNA array data was transformed into a heat map of gene expression in H1299 NSCLC cells stably expressing SETDB1: green and red represent down- and up-regulation of gene expression, respectively; a total of 711 genes were down-regulated and 522 genes were up-regulated after over-expression of SETDB1. (B) Pathway enrichment analysis between SETDB1 and control GFP-over-expressing H1299 cells using the KEGG database: the top 10 significantly enriched pathways are presented in the graph (\(p \leq 0.05; \ast \ p \leq 0.01; \ast \ast \ p \leq 0.001; \ast \ast \ast \ p \leq 0.0001\)). (C) RT–PCR validation of expression of genes related to the WNT signalling pathway in SETDB1 over-expressing H1299 cells; relative mRNA amounts were normalized to \(\beta\)-actin. (D) Recruitment of SETDB1 to the promoter region of APOE and IGFBP4 was examined by ChIP; nucleotide segments 2–2.3 kb upstream and 600–1000 bp downstream from the starting site (ATG) were divided into 14 (APOE) and 10 (IGFBP4) regions; enrichment of each fragment (250–300 bp) was examined by PCR. Enrichment occurred in the promoter regions of PA 3, PA 7, PA 10 and PA 14 of the APOE promoter (left panel) and regions of PI 2, PI 5, PI 6 and PI 8 of the IGFBP4 promoter (right panel): TSS, transcriptional start site; ATG, protein start codon; negative results of promoter regions PA 8 (APOE) and PI 7 (IGFBP4) are shown as controls for the respective genes. (E) mRNA levels of IGFBP4, APOE and \(\beta\)-catenin in SETDB1-silenced H1299 cells; relative mRNA amounts were normalized to \(\beta\)-actin; error bars indicate SD (\(n=4\)).

SETDB1 is a methyltransferase transferring a methyl group to the histone 3 lysine 9 (H3K9), which is generally believed to suppress gene expression associated with heterochromatin formation [16,33]. Recently, SETDB1 was identified as a novel oncogene in a zebrafish melanoma model [34]. During the preparation and submission of our manuscript, another investigative group also noted an oncogenic effect of SETDB1 in human lung cancers [35]. Our study confirms their observations and further explores the mechanism by which SETDB1 has tumourigenic activity.

SETDB1 maintains embryonic stemness by controlling the expression of stem cell-related factors (\(OCT4\) and \(Nanog\)) [21]. However, SETDB1 did not affect melanomas.
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Figure 6. Protein levels of β-catenin in NSCLC cells with either stably over-expressed or silenced SETDB1. (A) Western analysis of β-catenin accumulation in NSCLC cell lines with either silenced or over-expressed SETDB1; Ctrl, sh-Scramble; sh1,2,3, three shRNAs targeted to SETDB1. (B) Relative TOP/FOP activities when over-expressing SETDB1 in H1299 cells. The stably forced expression of either SETDB1- or GFP-containing cells were transfected with either pGL-TOP or pGL-FOP luciferase–reporter constructs; luciferase activities were measured 72 h after the transfection and normalized to the corresponding co-transfected Renilla luciferase activity; data are shown as the ratio between TOP/FOP and Renilla; error bars represent SD of three independent experiments. (C) Silencing of IGFBP4, LRP8 or FZD1 reduced the WNT activity in H1299 NSCLC cells: cells stably infected with either IGFBP4, LRP8 or FZD1 were transfected with either TOP or FOP, as well as Renilla control vector, and the levels of WNT pathway activity were determined by TOP/FOP assay. (D) Cytoplasmic and nuclear fraction of H1299 cells stably over-expressing either GFP or SETDB1: cells were grown on 10 cm dishes to 50–70% confluence, and the cytoplasmic and nuclear proteins were prepared as described in Materials and methods; cytoplasmic α-tubulin and nuclear histone H3 were used as controls of protein fractionation. (E) Western analysis of alternations of downstream genes (c-MYC, Cyclin D1) of the WNT pathway in SETDB1 stably over-expressing H1299 NSCLC cells. (F) Effect of stable silencing of β-catenin on clonogenic growth of H1299 cells over-expressing either GFP (control) or SETDB1; values are expressed as percentages of GFP control cells; mean ± SD (three independent experiments); the silencing efficiency of β-catenin was determined by western blot (right). (G) Clonogenic growth of either GFP or SETDB1 forced the expression of H1299 cells after stable silencing of FZD1, LRP8 or IGFBP4; values are expressed as percentages of GFP control cells; the experiments were performed in triplicate and results are presented as mean ± SD. (H) Schematic illustration of the proposed signalling interaction of SETDB1 and the WNT pathway

the expression of these genes in our study of NSCLC cells (data not shown), but it had a profound effect on the WNT pathway. We examined, for the first time, expression of SETDB1 by IHC in a large cohort of well-annotated lung cancer samples (387 lung cancers versus 106 normal cases). SETDB1 was statistically higher (p < 0.0001) in NSCLC tumours than in normal lung tissue, as measured by both percentage positive cells and their staining intensity for SETDB1. This occurred in adenocarcinomas and large cell carcinomas and adenosquamous carcinomas of the lung, and the levels of SETDB1 increased with increased grade of NSCLC.

WNT–β-catenin signalling plays a well-defined oncogenic role in colon and skin cancers [36]. Studies of both normal intestinal cells and colorectal cancer cells have shown that inhibition of WNT signalling exhausts normal intestinal cells and blocks the growth of colorectal cancer cells [37,38]. In addition, the WNT signalling pathway, including β-catenin, can also regulate the length of telomeres by directly controlling the transcription of telomerase reverse transcriptase, which further emphasizes the critical role the WNT pathway has in regulating the cell cycle, cell division and 'stemness' [39–41]. The WNT–β-catenin pathway plays an oncogenic role in NSCLC [36]. However, unlike in colorectal cancer, which has high levels of
β-catenin as a result of mutational loss of the adenomatous polyposis coli (APC) gene, mutation of either APC or β-catenin is infrequent in lung cancers [42]. Our studies suggest that activation of the WNT pathway by SETDB1 in lung cancer results in the accumulation of nuclear β-catenin, causing a transformation phenotype (Figure 6H).

As a H3K9 methyltransferase, SETDB1 has been characterized as a classic transcriptional repressor [21]. However, our microarray data showed that after induction of expression of SETDB1 in NSCLC, 711 genes transcriptionally decreased and another 522 genes increased in their expression. This up-regulation may represent secondary events as a result of down-regulation of repressive genes by SETDB1; or SETDB1 may enhance the expression of selected genes by unexplored mechanisms. Providing weight for the latter view, clear enrichment of SETDB1 on the promoter of a SETDB1-induced gene (IGFBP4) was noted. Indeed, studies by other researchers found that H3K9me3 markers are also presented in transcriptionally active genes [43–45]. For example, in a study of histone modification associated with the human X chromosome, H3K9me3 was found prominently within the actively transcribed genes, and the highest levels of H3K9me3 occurred within the highly expressed genes [46]. In addition, evidence from Drosophila showed that SETDB1 could both repress as well as activate genes; and this dual function was determined by the binding position of SETDB1 on the chromatin [46]. Notably, G9A, another H3K9 methyltransferase, also can enhance gene expression by acting as a transcriptional co-activator of the hormone receptor signalling pathways [47,48]. A similar activation effect also has been observed in the H3K27 methyltransferase EZH2 [49].

SETDB1 does not contain a DNA-binding motif, but can form a complex with KAP1 (Trim28) and HP1 or certain zinc finger proteins, which contain a DNA-binding motif resulting in DNA binding of the complex [14]. Adding to this complexity, SETDB1 can also bind to SuV39H1, G9a and GLP, which together can recruit additional factors to modulate transcription [50]. Further experiments are ongoing to elucidate the detailed mechanism of activation of genes by SETDB1. In addition, we made an unexpected observation – SETDB1 can lower the expression of P53; likewise, P53 can decrease the expression of SETDB1. Silencing of either one of these genes can reciprocally enhance the expression of another gene. Therefore, SETDB1 may further enhance cellular growth when silencing P53. This novel interaction requires further study.

In summary, we showed that some of the NSCLC samples have elevated SETDB1 expression associated with an increased grade of tumour. The increased levels of SETDB1 produced an increased clonogenic growth, associated with the activation of the WNT pathway and tumour growth. Our findings suggest that inhibitors that therapeutically target SETDB1 may benefit the large population of NSCLC patients whose tumours have high expression of SETDB1.

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Author contributions

QYS and LWD conceived and carried out the experiments, analysed the data and wrote the manuscript; JFX carried out the experiments; WC, SLL, NH, XYL, LX, TH and DCL analysed the data; LG, DC, VM, MA, SRK, NBD and JWS carried out the IHC experiments; SS and DX carried out the RT–PCR experiment in lung cancer patient samples; LZL and HY analysed the microarray expression data; and HPK conceived the experiments, analysed the data and wrote and reviewed the manuscript.

References

1. Soda M, Choi YL, Enomoto M, et al. Identification of the transforming EML4–ALK fusion gene in non-small-cell lung cancer. Nature 2007; 448: 561–566.
2. Crino L, Weder W, van Meerbeeck J, et al. Early stage and locally advanced (non-metastatic) non-small-cell lung cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol 2010; 21(suppl 5): v103–115.
3. Bell DW, Brannigan BW, Matsuo K, et al. Increased prevalence of EGFR-mutant lung cancer in women and in East Asian populations: analysis of estrogen-related polymorphisms. Clin Cancer Res 2008; 14: 4079–4084.
4. Shi Y, Au JS, Thongprasert S, et al. A prospective, molecular epidemiology study of EGFR mutations in Asian patients with advanced non-small-cell lung cancer of adenocarcinoma histology. J Thorac Oncol 2014; 9: 154–162.
5. Mano H, Takeuchi K. EML4–ALK fusion in lung. Am J Pathol 2010; 176: 1553–1554.
6. Soda M, Takada S, Takeuchi K, et al. A mouse model for EML4–ALK-positive lung cancer. Proc Natl Acad Sci USA 2008; 105: 19893–19897.
7. Bhaumik SR, Smith E, Shilatifard A. Covalent modifications of histones during development and disease pathogenesis. Nat Struct Mol Biol 2007; 14: 1008–1016.
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8. Shilatifard A. Chromatin modifications by methylation and ubiquitination: implications in the regulation of gene expression. Annu Rev Biochem 2006; 75: 243–269.

9. Strahl BD, Allis CD. The language of covalent histone modifications. Nature 2000; 403: 41–45.

10. Margueron R, Trojer P, Reinberg D. The key to development: interpreting the histone code?Curr Opin Genet Dev 2005; 15: 163–176.

11. Shahbazian MD, Grunstein M. Functions of site-specific histone acetylation and deacetylation. Annu Rev Biochem 2007; 76: 75–100.

12. Miao F, Natarajan R. Mapping global histone methylation patterns in the coding regions of human genes. Mol Cell Biol 2005; 25: 4650–4661.

13. Kouzarides T. Histone methylation in transcriptional control. Curr Opin Genet Dev 2002; 12: 198–209.

14. Schultz DC, Ayyanathan K, Negorev D, et al. SETDB1: a novel KAP-1-associated histone H3 lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRB zinc-finger proteins. Genes Dev 2002; 16: 919–932.

15. Loyola A, Tagami H, Bonaldi T, et al. The HP1α-CAF1-SETDB1-containing complex provides H3K9me1 for SuV39-mediated H3Kme3 in pericentric heterochromatin. EMBO Rep 2009; 10: 769–775.

16. Matsu T, Leung D, Miyashita H, et al. Provilal silencing in embryonic stem cells requires the histone methyltransferase ESET. Nature 2010; 464: 927–931.

17. Hayano T, Garg M, Yin D, et al. SOX17 is down-regulated in lung cancer. J Exp Clin Cancer Res 2013; 32: 17.

18. Shi S, Deng YZ, Zhao JS, et al. RACK1 promotes non-small-cell lung cancer tumorigenesis through activating sonic hedgehog signaling pathway. J Biol Chem 2012; 287: 7845–7858.

19. Mah V, Seligson DB, Li A, et al. Aromatase expression predicts survival in women with early-stage non-small cell lung cancer. Cancer Res 2007; 67: 10484–10490.

20. Huerta-Yepez S, Yoon NK, Hernandez-Cueto A, et al. Expression of phosphorylated Raf kinase inhibitor protein (pRIPK) is a predictor of lung cancer survival. BMC Cancer 2011; 11: 259.

21. Bilodeau S, Kagey MH, Frampton GM, et al. SETDB1 contributes to repression of genes encoding developmental regulators and maintenance of ES cell state. Genes Dev 2009; 23: 2484–2489.

22. Holland JD, Klaus A, Garratt AN, et al. Wnt signaling in stem and cancer stem cells. Curr Opin Cell Biol 2013; 25: 254–264.

23. Reya T, Clevens H. Wnt signalling in stem cells and cancer. Nature 2005; 434: 843–850.

24. Caruso A, Motolese M, Iacovelli L, et al. Inhibition of the canonical Wnt signalling pathway by apolliprotein E4 in PC12 cells. J Neurocytol 2006; 58: 361–371.

25. Pencheva N, Tran H, Buss C, et al. Convergent Multi-miRNA Targeting of ApoE drives LRPI/LRPS-dependent melanoma metastasis and angiogenesis. Cell 2012; 151: 1068–1082.

26. Ueno K, Hirata H, Majid S, et al. IGFBP-4 activates the Wnt/β-catenin signaling pathway and induces M-CAM expression in human renal cell carcinoma. Int J Cancer 2011; 129: 2360–2369.

27. Zhang J, Zhang X, Zhang L, et al. LRPS mediates Wnt/β-catenin signaling and controls osteoblast differentiation. J Bone Miner Res 2012; 27: 2065–2074.

28. Flahaut M, Meier R, Coulon A, et al. The Wnt receptor Fzd1 mediates chemoresistance in neuroblastoma through activation of the Wnt/β-catenin pathway. Oncogene 2009; 28: 2245–2256.

29. Barolo S. Transgenic Wnt/TGF pathway reporters: all you need is Let? Oncogene 2006; 25: 7505–7511.

30. Barker N, Clevens H. Mining the Wnt pathway for cancer therapeutics. Nat Rev Drug Discov 2006; 5: 997–1014.

31. Dvory-Sobol H, Sagiv E, Kazarov D, et al. Targeting the active β-catenin pathway to treat cancer cells. Mol Cancer Ther 2006; 5: 2861–2871.

32. Nicholas S, Tolwinski, Wieschaus E. A nuclear function for Armadillo/β-catenin. PLoS Biol 2004; 2: 8.

33. Karimi MM, Goyal P, Makasokva IA, et al. DNA methylation and SETDB1/H3K9me3 regulate predominately distinct sets of genes, retroelements, and chimeric transcripts in mESCs. Cell Stem Cell 2011; 8: 676–687.

34. Celci C, Houvras Y, Jane-Valbuena J, et al. The histone methyltransferase SETDB1 is recurrently amplified in melanoma and accelerates its onset. Nature 2011; 471: 513–517.

35. Rodriguez-Paredes M, de Paz AM, Simó-Ruialdalbas L, et al. Gene amplification of the histone methyltransferase SETDB1 contributes to human lung tumorigenesis. Oncogene 2013; 33: 2807–2813.

36. Dodde R, Brablett B. Wnt/β-catenin signaling in cancer stemness and malignant behavior. Curr Opin Chem Biol 2007; 19: 150–158.

37. Hirata A, Utikal J, Yamashita S, et al. Dose-dependent roles for canonical Wnt signalling in de novo crypt formation and cell cycle properties of the colonic epithelium. Development 2013; 140: 66–75.

38. de Sousa EM, Vermeulen L, Richel D, et al. Targeting Wnt signalling in colon cancer stem cells. Clin Cancer Res 2011; 17: 647–653.

39. Hoffmeyer K, Raggioli A, Rudloff S, et al. Wnt/β-catenin signalling regulates telomerase in stem cells and cancer cells. Science 2012; 336: 1549–1554.

40. Zhang Y, Toh L, Lau P, et al. Human telomerase reverse transcriptase (hTERT) is a novel target of the Wnt/β-catenin pathway in human cancer. J Biol Chem 2012; 287: 32494–32511.

41. Zhao X, Malhotra GK, Lele SM, et al. Telomerase-immortalized human mammary stem/progenitor cells with ability to self-renew and differentiate. Proc Natl Acad Sci USA 2010; 107: 14146–14151.

42. He B, Barg RN, You L, et al. Wnt signaling in stem cells and non-small-cell lung cancer. Clin Lung Cancer 2005; 7: 54–60.

43. Brinkman AB, Roelofsen T, Pennings SW, et al. Histone modification patterns associated with the human X chromosome. EMBO Rep 2006; 7: 628–634.

44. Vakoc CR, Mandat SA, Olenchock BA, et al. Histone H3 lysine 9 methylation and HP1β are associated with transcription elongation through mammalian chromatin. Mol Cell 2005; 19: 381–391.

45. Hahn MA, Wu X, Li AX, et al. Relationship between gene body DNA methylation and intragenic H3K9me3 and H3K36me3 chromatin marks. Proc Natl Sci One 2011; e18844.

46. Lundberg LE, Stenberg P, Larsson J. HP1α, Suv39h1/Suv39h2, and POF stimulate or repress gene expression depending on genomic position, gene length and expression pattern in Drosophila melanogaster. Nucleic Acids Res 2013; 41: 4481–4494.

47. Bittencourt D, Wu D-Y, Jeong KW, et al. G9a functions as a molecular scaffold for assembly of transcriptional coactivators on a subset of glucocorticoid receptor target genes. Proc Natl Acad Sci USA 2012; 109: 19673–19678.

48. Purcell DJ, Jeong KW, Bittencourt D, et al. A distinct mechanism for coactivator versus corepressor function by histone methyltransferase G9a in transcriptional regulation. J Biol Chem 2011; 286: 41963–41971.

49. Lee ST, Li Z, Wu Z, et al. Context-specific regulation of NF-kB target gene expression by EZH2 in breast cancers. Mol Cell 2011; 43: 798–810.

50. Fritsch L, Robin P, Mathieu JR, et al. A subset of the histone H3 lysine 9 methyltransferases Suv39h1, G9a, GLP, and SETDB1 participate in a multimeric complex. Mol Cell 2010; 37: 46–56.
SUPPLEMENTARY MATERIAL ON THE INTERNET

The following supplementary material may be found in the online version of this article:
Supplementary materials and methods
Figure S1. Testing the specificity of the SETDB1 antibody
Figure S2. SETDB1 copy number alternations and mutation status in different types of cancer
Figure S3. Effect of silencing β-catenin, FZD1, LRP8 or IGFBP4 on the proliferation of H1299 with over-expression of either GFP or SETDB1
Table S1. List of the short hairpin RNA (shRNA) target sequences used in this study
Table S2. Mutational genetic background of the NSCLC cells
Table S3. Primers used for the CHIP experiments

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