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ORIGINAL ARTICLE

The neuronal repellent SLIT2 is a target for repression by EZH2 in prostate cancer

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The neuronal repellent SLIT2 is repressed in a number of cancer types primarily through promoter hypermethylation. SLIT2, however, has not been studied in prostate cancer. Through genome-wide location analysis we identified SLIT2 as a target of polycomb group (PcG) protein EZH2. The EZH2-containing polycomb repressive complexes bound to the *SLIT2* promoter inhibiting its expression. SLIT2 was downregulated in a majority of metastatic prostate tumors, showing a negative correlation with EZH2. This repressed expression could be restored by methylation inhibitors or EZH2-suppressing compounds. In addition, a low level of SLIT2 expression was associated with aggressive prostate, breast and lung cancers. Functional assays showed that SLIT2 inhibited prostate cancer cell proliferation and invasion. Thus, this study showed for the first time the epigenetic silencing of SLIT2 in prostate tumors, and supported SLIT2 as a potential biomarker for aggressive solid tumors. Importantly, PcG-mediated repression may serve as a precursor for the silencing of SLIT2 by DNA methylation in cancer.
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Introduction

SLIT2, a human homolog of the *Drosophila Slit2* gene, belongs to the SLIT family of large secreted proteins, which also includes SLIT1 and SLIT3. The SLIT proteins are evolutionary conserved and contain an N-terminal signal peptide, four leucine-rich tandem repeats, seven or nine epidermal growth factor repeats, a laminin G domain and a C-terminal cysteine knot (Rothberg *et al*., 1988). A critical role of the SLIT proteins is to function as chemorepellents on navigating axons and migrating neurons during development (Wong *et al*., 2002). It mediates the repulsive cues and guides away the projection of normal axons and developing neurons (Brose *et al*., 1999). Although SLIT1 is predominantly expressed in the nervous system, SLIT2 is also expressed in non-neuronal tissues such as lung, breast, kidney and heart, suggesting innovative roles in addition to axon guidance (Wu *et al*., 2001).

Emerging evidence suggests that, outside the nervous system, the neuronal repellent SLIT2 inhibits the migration of a number of other cell types towards a variety of chemotactic factors. For example, SLIT2 inhibits CXCL12-induced chemotaxis of leukocytes (Wu *et al*., 2001) and transendothelial migration of T cells (Prasad *et al*., 2007). In breast cancer cells, SLIT2 mediates inhibition of CXCR4-induced chemotaxis, chemoinvasion and adhesion, thus implicating a role in preventing tumor metastasis (Prasad *et al*., 2004). The tumor-suppressor activities of SLIT2 have been reported in a number of cancer types (Tseng *et al*., 2010). Both SLIT2 overexpression and SLIT2-containing conditioned medium have been shown to suppress breast cancer cell growth *in vitro* (Dallol *et al*., 2002). In medulloblastoma, SLIT2 inhibits cell invasion by interfering with the CDC42 and RAC1 pathways (Werbowetski-Ogilvie *et al*., 2006). More recently, SLIT2 was shown to increase apoptosis, decrease cell proliferation, and inhibit cell migration and invasion *in vitro* in squamous cell carcinoma and fibrosarcoma (Kim *et al*., 2008). In addition, overexpression of SLIT2 suppresses xenograft tumor growth and inhibits the metastasis of tumor cells after intravenous inoculation in nude mice, providing compelling evidence for *SLIT2* as a tumor suppressor gene (Kim *et al*., 2008; Prasad *et al*., 2008).

Consistent with its tumor suppressor activity, *SLIT2* was shown to be downregulated, primarily through DNA hypermethylation, in a number of cancer types.

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The CpG islands in the promoter region of the *SLIT2* gene were hypermethylated in 59% of gliomas with concordant downregulated expression (Dallol *et al*., 2003a). In another study, promoter hypermethylation of *SLIT2* was shown in 29% of neuroblastomas, 38% of Wilms' tumors and 25% of renal cell carcinomas (Astuti *et al*., 2004). Frequent epigenetic silencing of *SLIT2*, with corresponding decrease in SLIT2 expression, has also been reported in a majority of lymphocytic leukemias (Dunwell *et al*., 2009), 72% of primary colorectal cancers (Dallol *et al*., 2003b), 83.3% of primary hepatocellular carcinomas (Jin *et al*., 2009) and almost all lung adenocarcinomas (Dammann *et al*., 2005b). In addition, SLIT2 promoter hypermethylation was detected in 59% of breast cancers and, importantly, in their paired serum DNA, suggesting its potential as a noninvasive biomarker (Dallol *et al*., 2002; Sharma *et al*., 2007). SLIT2 expression and function, however, have not been studied in prostate tumors.

Polycomb group (PcG) proteins are transcriptional repressors that inhibit developmental regulators in embryonic stem cells and silence tumor suppressor genes in cancer (Mathews *et al*., 2009). They function through multimeric chromatin-associated polycomb repressive complexes, including PRC1 and PRC2. The core components of PRC1 include Bmi1, Ring1, Ring2 and HPC2, while PRC2 is mainly composed of SUZ12, EED and EZH2, with EZH2 enzymatically catalyzing the methylation of lysine 27 of histone H3 (3mH3K27) (Simon and Kingston, 2009). EZH2 is a *bona fide* oncogene that mediates cancer cell proliferation and invasion and is frequently found to be upregulated in a number of cancer types, including prostate and breast cancers (Varambally *et al*., 2002). EZH2 is thought to promote tumorigenesis through epigenetic silencing of a group of tumor suppressor genes, including *ADRB2*, *CDH1*, *PSP94* and *DAB2IP* (Chen *et al*., 2005; Beke *et al*., 2007; Yu *et al*., 2007b; Cao *et al*., 2008). However, a majority of polycomb target genes in cancer cells remain unknown.

In this study, genome-wide location analysis of prostate cancer cells revealed *SLIT2* as a top target gene of EZH2-mediated H3K27 trimethylation. We show that SLIT2 is downregulated in prostate cancer by epigenetic mechanisms and represents a potent prognostic biomarker that merits further evaluation in large patient cohorts. In addition, overexpression of SLIT2 inhibits prostate cancer cell proliferation and invasion. Our study is the first to show epigenetic silencing of SLIT2 in prostate cancer and establishes a novel mechanism for SLIT2 repression in cancer involving PcG proteins, suggesting that PcG-mediated chromatin change may in general serve as a precursor for the silencing of tumor suppressor genes by DNA methylation.

Results

SLIT2 is a target of EZH2-mediated H3K27 trimethylation in prostate cancer

To investigate the target genes of PcG proteins in prostate cancer, we performed genome-wide location analysis of SUZ12 and 3mH3K27 in the LNCaP prostate cancer cells (Figure 1a). Out of approximately 80 000 probes present on the promoter array, 7326 showed significant enrichment ($P<0.0001$) in the chromatin immunoprecipitation assay (ChIP) sample relative to the whole-cell extract. There were only 15 probes with more than 10-fold enrichment, out of which two mapped to the regulatory regions of the same gene, *SLIT2*. To evaluate the reproducibility of the assay, ChIP-on-chip of 3mH3K27 was replicated using two independent ChIP-enriched DNA fragments. Importantly, the enrichment ratios from both experiments were highly reproducible, with $r^2=0.88$ (Figure 1b). *SLIT2* remained among the most enriched targets of 3mH3K27 in both replicates, and all five probes within the *SLIT2* promoter region ranked among the top 5% most-enriched targets of SUZ12 and 3mH3K27 (Supplementary Figure S1).

To confirm these genome-wide location data, we applied ChIP-PCR, which couples the conventional ChIP assay with quantitative PCR (qPCR) using gene-specific primers to examine polycomb occupancy on the *SLIT2* promoter. ChIP was performed in the LNCaP cells using antibodies against EZH2, SUZ12 and 3mH3K27. Importantly, our data showed 1.6-, 4.7- and 21.5-fold of enrichment by EZH2, SUZ12 and 3mH3K27, respectively, thus confirming *SLIT2* as a target of PRC2 (Figure 1c). The difference in fold enrichment largely reflects the quality of the antibodies for ChIP experiments. This repressive 3mH3K27 mark can be effectively reduced by histone deacetylase inhibitor SAHA (Supplementary Figure S2), which is consistent with the notion that EZH2-mediated H3K27 methylation requires histone deacetylase activity (van der Vlag and Otte, 1999). As PRC2 binding is known to recruit PRC1, leading to a widespread 3mH3K27 (Sparmann and van Lohuizen, 2006), we tested whether PRC1 binds to the SLIT2 promoter. Interestingly, ChIP-PCR using antibodies against PRC1 proteins BMI1, RING1 and RING2 revealed significant enrichment at the SLIT2 promoter (Supplementary Figure S3). To determine whether this protein–DNA interaction holds true *in vivo*, we performed ChIP analysis of 3mH3K27 in one localized prostate cancer and three metastatic prostate tumors. Importantly, the SLIT2 promoter contained strong 3mH3K27 modification, especially in metastatic prostate tumors (Figure 1d). Taken together, our data show that SLIT2 recruits PRC2 and PRC1 complex proteins, resulting in nucleosomes harboring repressive histone marks.

SLIT2 expression is negatively regulated by EZH2

To investigate the consequence of recruiting polycomb repressive complexes to the *SLIT2* promoter, we tested the level of SLIT2 expression following EZH2 deregulation *in vitro*. As EZH2 is expressed at low levels in benign cells and is primarily upregulated in aggressive tumors, we overexpressed EZH2 in several benign prostate and breast cell lines, including PrEC, RWPE, H16N2 and HME. Importantly, consistent with its

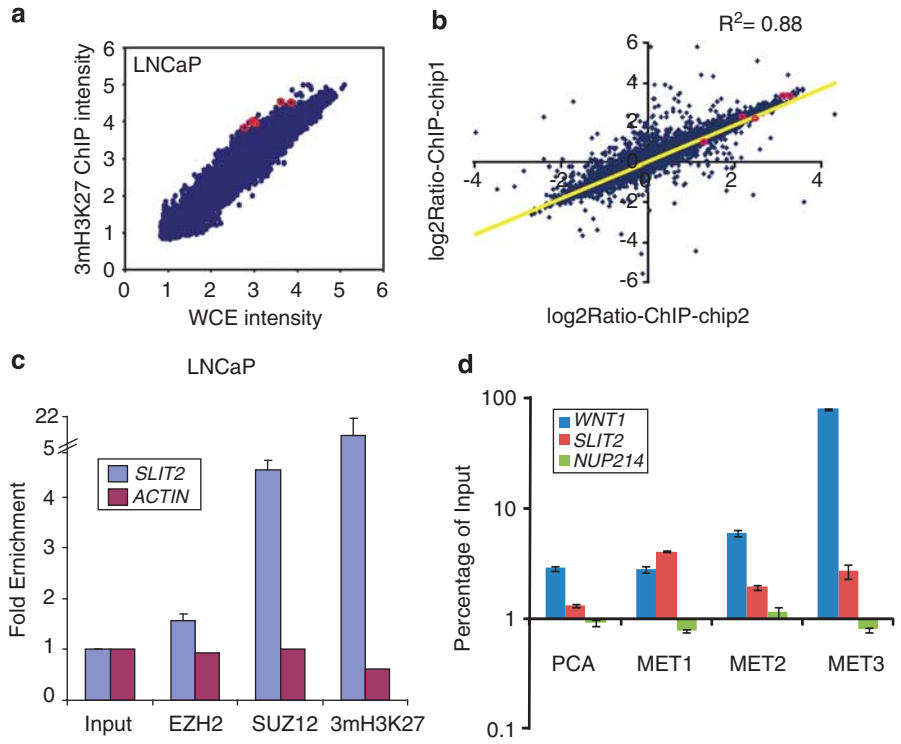

Figure 1 PRC2 binds to the SLIT2 promoter leading to H3K27 trimethylation. (**a**) ChIP-on-chip analysis of 3mH3K27 in LNCaP prostate cancer cells. ChIP-on-chip analysis was done using Agilent promoter arrays and analyzed by Agilent ChIP analytics software. The scatter plot shows ChIP-enriched intensity (processed signals) relative to control whole-cell extract (WCE) for all genes in log10 scale. Red circles highlight the probes corresponding to the SLIT2 promoter. (**b**) Replicate ChIP-on-chip analysis of 3mH3K27 shows high correlation. Replicate ChIP-on-chip hybridization was performed using DNA sample independently enriched by an anti-3mH3K27 antibody in LNCaP prostate cancer cells. Log2 ratio of ChIP-enriched over WCE intensity from each experiment was plotted. Red circles highlight the probes corresponding to the SLIT2 promoter. (**c**) The *SLIT2* promoter is occupied by the PRC2 proteins and trimethylated at H3K27. ChIP assay was performed in LNCaP using antibodies against EZH2, SUZ12 and 3mH3K27. qPCR was performed to evaluate enrichment of *SLIT2* over the input WCE DNA. *ACTIN* was used as a negative control gene for PRC2 binding. (**d**) One localized and three metastatic prostate cancer tissues were subjected to ChIP using anti-3mH3K27 antibody. ChIP-enriched DNA and the input DNA were first amplified through a ligation-mediated PCR. Equal amounts (50 ng) of amplified ChIP DNA and the input DNA were then subjected to PCR, and enrichment by ChIP was assessed relative to the input DNA. Error bar: $n = 3$, mean ± s.e.m. The WNT1 promoter and the intragenic region of NUP214 were used as positive and negative controls, respectively.

binding by the PRC2 complex, *SLIT2* was significantly downregulated by *EZH2* overexpression in all the four cell lines (Figure 2a). To confirm that this regulation holds true at the protein level, we performed an immunoblot analysis of SLIT2 and EZH2 in the RWPE and H16N2 cells infected with EZH2 overexpressing adenovirus. Our results showed clear repression of the SLIT2 protein following EZH2 overexpression (Figure 2b). Next, we performed RNA interference of EZH2 in metastatic prostate cancer cell lines DU145 and PC3, and achieved, respectively, 1.7- and 5.2-fold reduction in *EZH2* expression. Concordantly, *SLIT2* expression was significantly derepressed, leading to a 1.8- and 2.9-fold increase in DU145 and PC3 cells, respectively (Figure 2c and Supplementary Figure S4). It is important to note that the level of EZH2 knockdown and the response of SLIT2 expression to EZH2 knockdown vary between cell lines, probably because of other regulatory mechanisms specific to each cell type.

As histone deacetylase inhibitor SAHA reduces the repressive 3mH3K27 mark on the SLIT2 promoter, we examined the level of SLIT2 expression after SAHA treatment. Interestingly, *SLIT2* was markedly derepressed by SAHA by approximately 9.7-, 3.0- and 1.4-fold in LNCaP, PC3 and DU145 cells, respectively (Figure 3a). This derepression is not because of the non-specific effects of SAHA on cell cycle arrest and presumably less DNA/protein synthesis (Supplementary Figure S5). Previous studies have identified a PRC2-inhibiting compound, 3-deazaneplanocin A, that is able to derepress EZH2 target genes (Tan *et al*., 2007). We thus tested whether 3-deazaneplanocin A is able to inhibit EZH2-mediated repression of SLIT2. Interestingly, our results showed a marked upregulation of *SLIT2* after 3-deazaneplanocin A treatment in breast and prostate cancer cell lines, including MDA-MB-231, SKBR3, DU145 and LNCaP (Figure 3b). Therefore, SLIT2 is a target of EZH2-mediated transcriptional repression and can be reactivated by PRC2 inhibitors.

SLIT2 promoter hypermethylation in prostate cancer

As hypermethylation of the CpG islands in the *SLIT2* promoter has been a classical mechanism for *SLIT2*

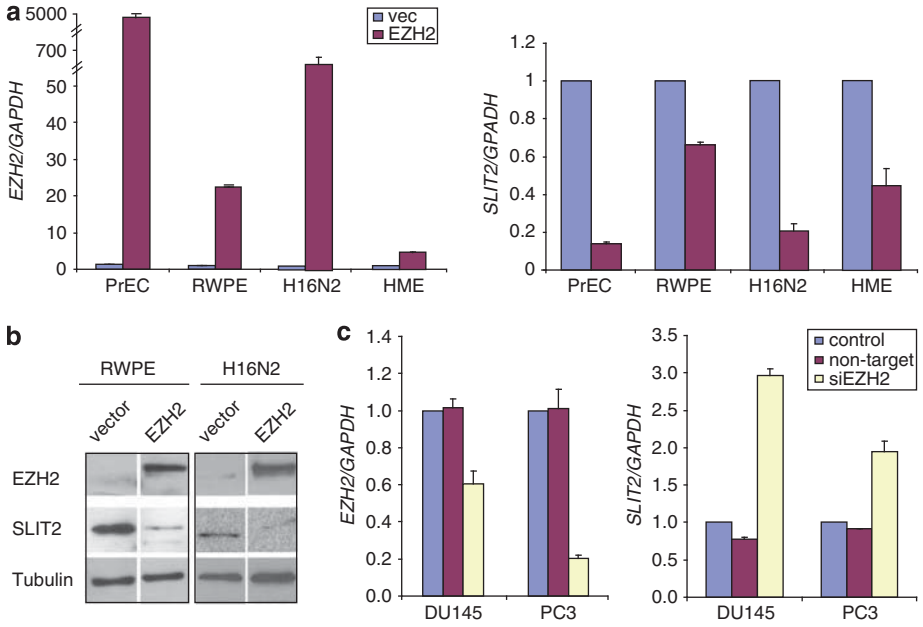

Figure 2 *SLIT2* expression is negatively regulated by EZH2. (**a**) *EZH2* overexpression represses the transcript levels of *SLIT2*. Benign immortalized prostate cell lines, PrEC and RWPE, and breast cell lines, H16N2 and HME, were infected with an adenovirus overexpressing EZH2 or with an empty vector, and analyzed for *EZH2* and *SLIT2* mRNA levels by qRT–PCR. (**b**) Immunoblot analysis of EZH2 and SLIT2 in benign immortalized prostate cell line (RWPE) and breast cell line (H16N2) after an infection with EZH2 adenovirus or vector control for 48 h. The β-tubulin protein was used as a loading control. (**c**) qRT–PCR analysis of *EZH2* and *SLIT2* transcripts in DU145 and PC3 prostate cancer cells after RNA interference of EZH2.

repression in cancer, we examined whether the *SLIT2* promoter is hypermethylated in prostate cancer cells. We first carried out ChIP experiments using an antibody specific to methylcytidines and performed qPCR analysis using primers specific to the *SLIT2* promoter. The promoter region of *IL3*, a previously reported methylation target bound by DNMT1 (Liu *et al*., 2005), was used as a positive control. Similar to IL3, the *SLIT2* promoter was substantially more enriched by the antibody against 5-methylcytidine than the immunoglobulin G (control) (Figure 4a).

As promoter hypermethylation can be reduced by the DNA methylation inhibitor 5-aza-2′-deoxycytidine leading to gene reactivation, we treated LNCaP and PC3 prostate cancer cells with 5-aza-2′-deoxycytidine and monitored the SLIT2 level. We used the A549 lung cancer cell line as a positive control, as previous studies have reported *SLIT2* promoter hypermethylation in almost all cases of lung adenocarcinoma (Dammann *et al*., 2005a). qRT–PCR analysis revealed that 5-aza-2′-deoxycytidine significantly derepressed SLIT2 in all the three cell lines tested, further supporting SLIT2 as a target of DNA hypermethylation (Figure 4b).

To determine the status of *SLIT2* promoter hypermethylation *in vivo*, we performed bisulfite sequencing of the *SLIT2* promoter region in a set of four benign prostate tissues, five localized and five metastatic prostate cancer tissues. Our results showed that the CpG islands in the *SLIT2* promoter were rarely methylated in benign samples but the level of hypermethylation markedly increased in localized and metastatic prostate tumors (Figure 4c).

SLIT2 is downregulated in metastatic prostate cancer

We have now shown that SLIT2 is a target for repression by EZH2-mediated histone methylation, as well as promoter hypermethylation in prostate cancer using *in vitro* cell line models. We next examined whether SLIT2 expression is downregulated in prostate tumors *in vivo*. We first analyzed the microarray profiling data sets of prostate cancer tissues by using Oncomine (http://www.oncomine.com). *SLIT2* was found to be significantly downregulated, in particular, in metastatic prostate cancer in multiple microarray cancer profiling data sets (Figure 5a). In addition, the expression levels of *SLIT2* and *EZH2* showed a highly significant ($r<-0.4$; $P<0.0001$) anti-correlation supporting *EZH2* repression of *SLIT2 in vivo* (Supplementary Figure S6). To confirm this, we performed qRT–PCR analysis of *SLIT2* and *EZH2* in a set of eight benign prostatic tissues, seven localized and seven metastatic prostate tumors. Concordantly, *SLIT2* was remarkably downregulated ($P<0.001$ by *t*-test) in metastatic prostate tumors (Figure 5b). Furthermore, immunoblot analysis of SLIT2 and EZH2 in a set of three benign, four localized and seven metastatic prostate tumors confirmed that the SLIT2 protein is highly expressed in benign tissues, decreased in localized and almost absent in metastatic prostate cancer tissues (Figure 5c). In contrast, EZH2 protein is overexpressed in metastatic prostate cancers. In addition, our recent RNA-Seq analysis of 8 benign, 15 localized prostate cancer and 20 metastatic prostate tumors likewise revealed negatively correlated ($r=-0.47$) expression of *EZH2* and *SLIT2* (Figure 5d). None of the 20

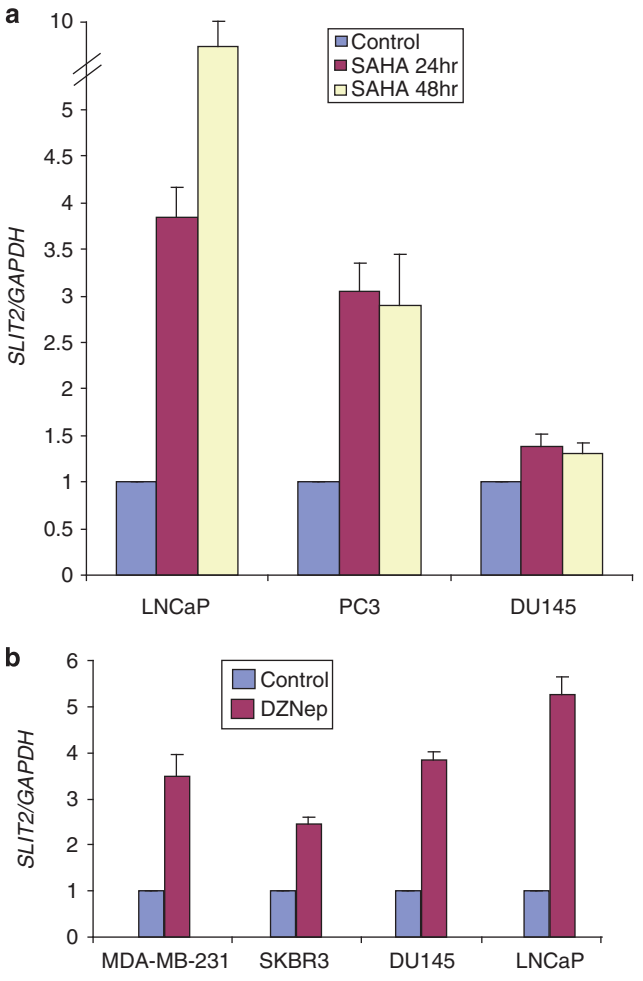

Figure 3 Inhibiting EZH2 function de-represses *SLIT2*. (**a**) *SLIT2* expression is upregulated by a histone deacetylase (HDAC) inhibitor SAHA. Prostate cancer cell lines LNCaP, PC3 and DU145 were treated with 5 μM of SAHA for 24 and 48 h, and analyzed by qRT–PCR. (**b**) Marked upregulation of SLIT2 by the PRC2-inhibiting compound 3-deazaneplanocin A (DZNep). Breast (MDA-MB-231 and SKBR3) and prostate (DU145 and LNCaP) cancer cells were treated with 5 μM DZNep for 48 h and analyzed by qRT–PCR. Expression of target gene was normalized to the amount of the *GAPDH* housekeeping gene.

metastatic prostate tumors expressed high levels of *SLIT2* (Figure 5e).

Low level of SLIT2 expression is associated with aggressive tumors

We next examined whether the expression level of SLIT2 is associated with the survival of prostate cancer patients by first exploring publically available microarray profiling data sets of localized prostate tumors with varying disease outcome (Glinsky *et al*., 2004; Yu *et al*., 2004). For each data set, primary prostate tumors were first classified into two groups based on the expression level of the *SLIT2* gene. Kaplan–Meier analysis was used to evaluate survival differences between the two groups and revealed that, for both data sets, the two groups differed significantly in clinical outcome ($P=0.025$ for the Glinsky *et al*. data set and $P=0.011$ for the Yu *et al*. data set) (Figure 6). As epigenetic silencing of SLIT2 has also been reported in lung and breast cancers, we evaluated its power in predicting the survival of lung and breast cancer patients. Similarly, Kaplan–Meier analyses showed that a low level of *SLIT2* expression was significantly associated with more aggressive disease in multiple cancer data sets, including the Raponi *et al*. (2006), van 't Veer *et al*. (2002), van de Vijver *et al*. (2002), Miller *et al*. (2005), Pawitan *et al*. (2005), Wang *et al*. (2005) and Oh *et al*. (2006) data sets.

To confirm this at the protein level, we performed tissue microarray analysis of SLIT2 in a cohort of 169 tumor cores from 79 patients. Univariate outcome analysis showed that the SLIT2 level is significantly ($P=0.04$) associated with the hazard of prostate-specific antigen (PSA) recurrence (Supplementary Table S1). Kaplan–Meier analysis of recurrence-free survival showed that lower level of SLIT2 protein is associated with poorer clinical outcomes in prostate cancer patients (Supplementary Figure S7). Multivariate outcome analysis also indicated a trend of SLIT2 in predicting the risk of PSA recurrence (Supplementary Table S2), thus suggesting that SLIT2 has some association with the clinical outcome, but not at an independent level.

To shed some light on the mechanisms underlying SLIT2's association with clinical outcome, we examined the role of SLIT2 in prostate cancer. As SLIT2 is a secreted protein, we obtained SLIT2-conditioned medium from HEK293 cells stably overexpressing SLIT2 (Figure 7a). Compared with the conditioned medium from control cells (HEK293/pBC), SLIT2-containing conditioned medium significantly inhibited LNCaP cell invasion (Figure 7b) and proliferation (Figure 7c).

Discussion

The PcG proteins were initially discovered for their roles in body patterning in *Drosophila* and have been shown to be critical to the cellular memory system, through which epigenetic modifications encode inheritable cellular identity (Kanno *et al*., 2008). More recently, PRC2 proteins including SUZ12 and EZH2 have been implicated in maintaining the pluripotency of embryonic stem cells by silencing developmental regulators (Lee *et al*., 2006). Deregulated expression of the PcG protein EZH2 has been documented in a variety of tumor types and is associated with poor clinical outcomes (Simon and Lange, 2008). The oncogenic properties of EZH2 were believed to be mediated by its epigenetic silencing of a group of tumor suppressor genes in cancer (Yu *et al*., 2007b). Identification of EZH2 target genes may facilitate the understanding of its function, as well as the discovery of novel biomarkers and therapeutic targets. In this study, we report neuronal repellent *SLIT2* as a novel target of EZH2-mediated epigenetic silencing in prostate cancer.

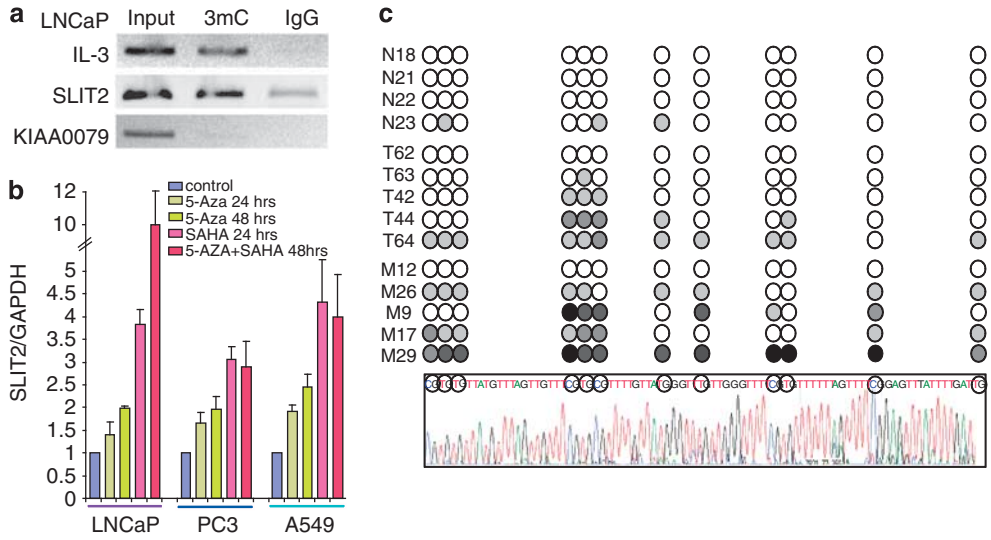

Figure 4 DNA methylation of SLIT2 promoter in prostate cancer. (**a**) The SLIT2 promoter was enriched by 5-methylcytidine. Methylated DNA precipitation was performed in the LNCaP cells using an anti-5-methylcytidine antibody and immunoglobulin G (control). The IL-3 gene was used as a positive control and KIAA0079 as a negative control. (**b**) Methylation inhibitors re-activate *SLIT2* expression. The LNCaP, PC3 and A549 cells were treated with 5 μM histone deacetylase inhibitor SAHA and/or 5 μM DNA methylation inhibitor 5-aza-2′-deoxycytidine for 24 or 48 h. Total RNA was isolated and analyzed by qRT–PCR. *SLIT2* expression was normalized to GAPDH. Error bars represent mean ± s.e.m. (**c**) Detection of SLIT2 promoter region by methylation in prostate tumor samples. DNA samples were bisulfite-modified and the SLIT2 promoter region was amplified. At least five clones per sample were subjected to bisulfite sequencing. The circles indicate the CpG dinucleotides shown in the sequence chromatograph. The shades of gray indicate detection of methylated CpG in 20, 40, 60, 80 or 100% of the clones. N is for normal, T for localized tumor and M for metastatic prostate tumors. An example sequence trace of one clone is shown at the bottom of the panel. Circled bases represent the CpG dinucleotides analyzed above.

SLIT2 functions as a neural repellent in the central nervous system, guiding axon elongation and branching through repulsive cues (Brose *et al*., 1999; Wang *et al*., 1999). During malignancy, it acts as a chemorepellent in multiple cancer types by inhibiting chemotaxis, cell migration and invasion, thus demonstrating the properties of tumor suppressor genes (Prasad *et al*., 2004). It is frequently downregulated in a variety of cancer types, including colorectal, lung and breast cancers. Hypermethylation of the CpG islands in the *SLIT2* promoter is a well-established mechanism for SLIT2 repression in tumors. In this study, we report, for the first time, a novel mechanism involving polycomb-mediated histone modification to epigenetic silencing of SLIT2 in prostate cancer. We found that PcG proteins bind to the SLIT2 promoter in prostate cancer cell lines as well as prostate tumors to inhibit its expression. This repressed expression can be reactivated by histone deacetylase inhibitors or EZH2-inhibiting compounds. In addition, we showed that DNA hypermethylation of the SLIT2 promoter is present in prostate cancer, similar to many other cancers. Importantly, our results support the recently formulated hypothesis that PcG-mediated repression may be a prerequisite step for the silencing of tumor suppressor genes by DNA hypermethylation (Sparmann and van Lohuizen, 2006; Ohm *et al*., 2007).

Although SLIT2 has been shown to be downregulated in a number of tumor types, it has, thus far, not been shown in the context of prostate cancer, despite prostate cancer being a leading cause of cancer-related death in American men. Our study provides a timely report that SLIT2 is repressed in prostate carcinoma and, in particular, in a majority of metastatic prostate tumors. This downregulated expression was observed in multiple microarray profiling data sets of prostate tumors and confirmed by qRT–PCR, RNA-Seq and immunoblot analysis. In addition, we show that the levels of SLIT2 expression are anti-correlated with those of EZH2. Importantly, while high levels of EZH2 are associated with aggressive prostate and breast cancers, low levels of SLIT2, in contrast, predict poor clinical outcomes. Interestingly, an earlier study of breast cancer has shown epigenetic silencing of SLIT2 in both breast cancer tissues and paired serum samples (Sharma *et al*., 2007). Therefore, in future studies, it would be of great interest to examine the expression of SLIT2 in the sera and urine of prostate cancer patients and determine its value as a non-invasive prognostic biomarker in prostate cancer.

In summary, through genome-wide location analysis of PcG proteins, we identified SLIT2 as a direct target of EZH2-mediated H3K27 trimethylation in prostate cancer. We also showed that the CpG islands in the *SLIT2* promoter are hypermethylated in prostate cancer, like in many other cancer types. Functional assays implicated the role of SLIT2 in inhibiting prostate cancer cell growth and invasion. In addition, our study suggests that SLIT2 may be a potent noninvasive prognostic biomarker of prostate cancer.

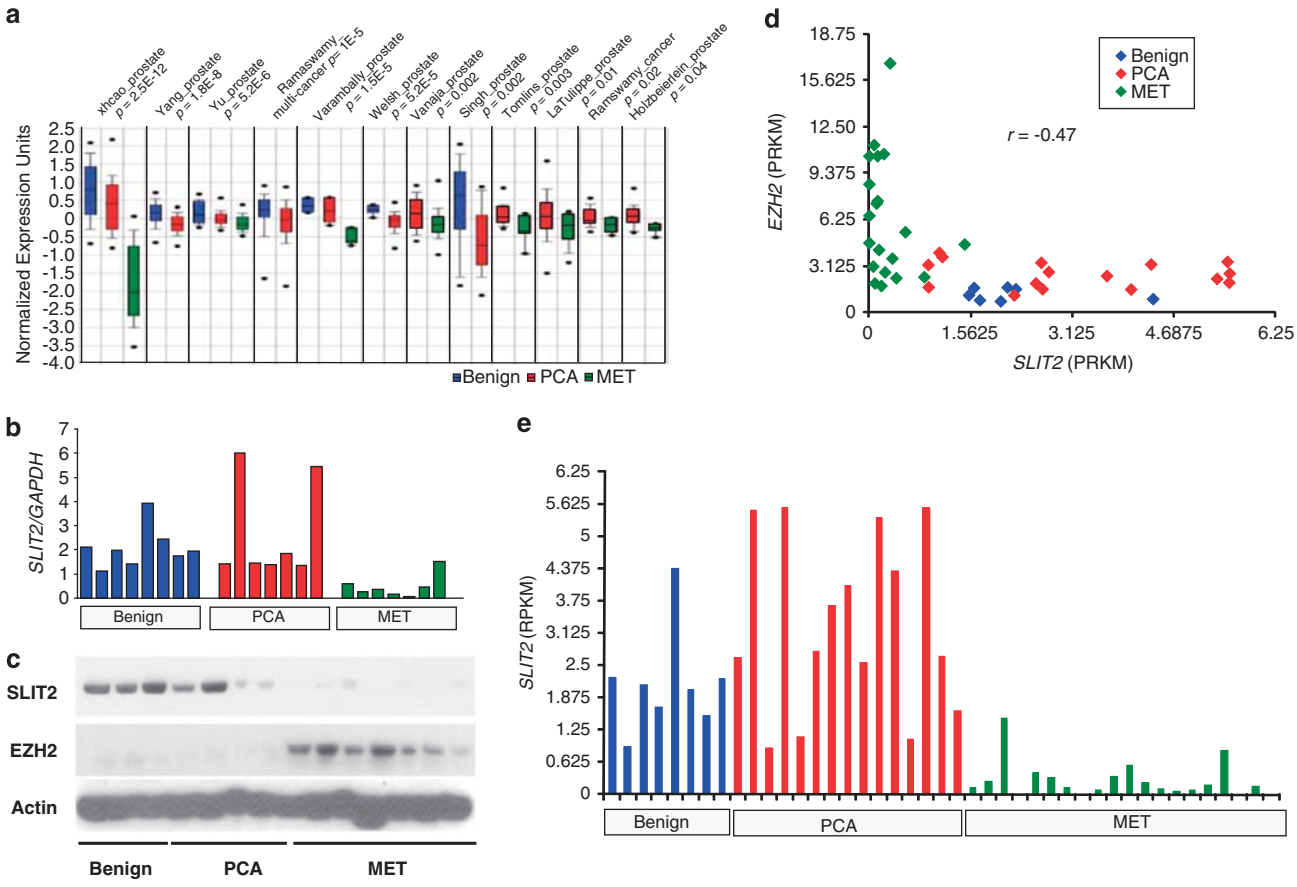

Figure 5 SLIT2 downregulation in prostate cancer. (**a**) *SLIT2* expression in multiple cancer microarray data sets available in the Oncomine (www.oncomine.com). (**b**) qRT–PCR analysis of *SLIT2* and *EZH2* transcripts in a cohort of eight benign prostate hyperplasia (Benign), seven localized prostate cancer (PCA) and seven metastatic prostate cancer (MET) tissues. Expression of target genes was normalized to the amount of the *GAPDH* housekeeping gene. *SLIT2* expression is markedly ($P = 0.0004$ by *t*-test) lower in metastatic prostate cancers. (**c**) The immunoblot of SLIT2 in a cohort of three benign prostate hyperplasia (Benign), four localized prostate cancer and seven metastatic prostate cancer tissues. (**d**) Negative correlation ($r = -0.47$) between the transcript levels of *EZH2* and *SLIT2* in a cohort of prostate cancer analyzed by RNA-Seq. The scatter plot of *EZH2* and *SLIT2* expression was shown from all the profiled samples by our in-house RNA-Seq analysis of 8 benign, 15 localized and 20 metastatic prostate tumors. Expression values are in RPKM (the number of mapable sequencing reads per million per kilobase of cDNA sequence) (**e**) SLIT2 expression (in RPKM) analyzed by RNA-Seq as in (**d**). SLIT2 expression is downregulated in metastatic prostate cancers ($P < 0.001$ by *t*-test).

Materials and methods

Cell culture

The prostate and breast cell lines were obtained from the American Type Culture Collection. LNCaP, DU145, PC3, MDA-MB-231 and SKBR3 cells were cultured in RPMI supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). The PrEC (Lonza, Conshohocken, PA, USA) and RWPE (ATCC, Manassas, VA, USA) cells were grown in their respective medium as specified by the manufacturer. HME and H16N2 cells were grown in Ham's F12 supplemented with 0.1% bovine serum albumin, 0.5 μg/ml fungizone, 5 μg/ml gentamycin, 5 mM ethanolamine, 10 mM HEPES, 5 μg/ml transferrin, 10 μM T3, 50 μM selenium, 5 μg/ml insulin, 1 μg/ml hydrocortisone and 10 ng/ml epidermal growth factor. HEK293 cells stably expression SLIT2 (HEK293/SLIT2) were cultured as previously reported (Wu *et al*., 2001) and grown in Dulbecco's Eagle's modified medium supplemented with 10% fetal bovine serum and 200 μg/ml G418 (Sigma-Aldrich, St Louis, MO, USA). For inhibitor treatment, prostate cancer cells were treated with 5 μM 3-deazaneplanocin A, 5 μM SAHA and/or 5 μM 5-aza-2′-deoxycytidine for the indicated length of time. EZH2 adenovirus infection and RNA interference were performed as previously described (Yu *et al*., 2007a). At 48 h after adenovirus infection or RNA interference, cells were harvested for RNA isolation using TRIzol (Invitrogen).

ChIP and ChIP-on-chip

ChIP and ChIP-on-chip were performed as previously described (Yu *et al*., 2007a). Antibodies (5 μg) used for ChIP included monoclonal anti-EZH2 (BD Biosciences, San Jose, CA, USA), polyclonal anti-SUZ12 (Millipore, Billerica, MA, USA) and polyclonal anti-3mH3K27 (Millipore) antibodies. ChIP-on-chip was done using the 44K, two-set Agilent proximal promoter arrays according to the manufacturer's protocols (Agilent, Santa Clara, CA, USA). The ChIP-on-chip data have been deposited to the NCBI GEO database with accession number GSE9069.

Bisulfite conversion and methylation analysis

DNA samples were bisulfite-modified before PCR amplification and sequencing according to previously described procedures with slight modifications (Dallol *et al*., 2003a). The region analyzed was upstream of the SLIT2 translation start site in accession number NT_006316. More precisely, the region PCR amplified was chr4: 19 863 915–19 864 324 and the

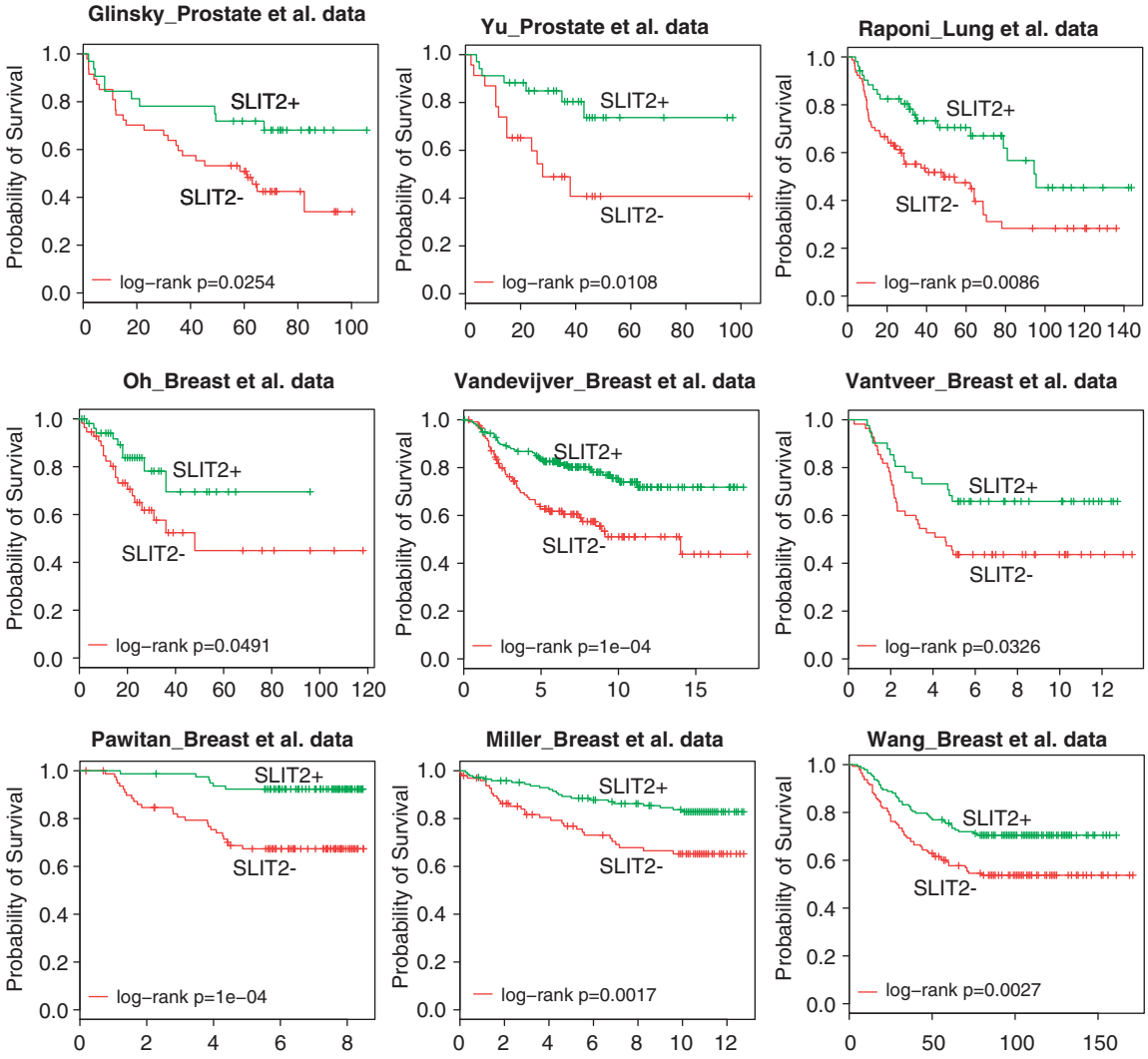

Figure 6 The expression level of *SLIT2* predicts clinical outcome in multiple cancer microarray profiling data sets. Samples of each data set were classified into two groups based on the transcript levels of *SLIT2*. The two groups were assessed for survival differences by Kaplan-Meier (KM) analysis. KM plots are shown for Glinsky *et al.* (2004), Yu *et al.* (2004) prostate cancer data sets, Raponi *et al.* (2006) lung cancer data sets and . van 't Veer *et al.* (2002), van de Vijver *et al.* (2002), Miller *et al.* (2005), Pawitan *et al.* (2005), Wang *et al.* (2005) and Oh *et al.* (2006) breast cancer data sets.

region sequenced was chr4: 1 863 964–19 864 050 according to the March 2006 NCBI Build 36.1. The primers used to PCR amplify the SLIT2 promoter regions were 5′-AGTTTAGAG TYGTGYGTTTTTAGAAT-3′ (forward) and 5′-CAAAAAC TCCTTAAACAACTTTAAATCCTAAAA-3′ (reverse). The PCR conditions were 95 °C for 10 min, followed by 40 cycles of 1 min denaturation at 95 °C, 1 min annealing at 54 °C and 2 min extension at 74 °C using Qiagen HotStarTaq DNA Polymerase (Qiagen, Valencia, CA, USA). The PCR products were cloned into the pGEM-T Easy system (Promega, Madison, WI, USA) before sequencing. For each sample, at least five clones were sequenced using the T7 vector primers. The methylation index is calculated as a percentage using the equation: number of CpG dinucleotides methylated/total number of CpG dinucleotides sequenced × 100.

Methylated DNA immunoprecipitation

Methylated DNA immunoprecipitation was performed in genomic DNA using an anti-5-methylcytidine antibody (BI-MECY_0100, Eurogentec, Fremont, CA, USA) or immunoglobulin G (control) (Santa Cruz, Santa Cruz, CA, USA) according to previously published protocols (Weber *et al.*, 2005). qPCR was used to determine the enrichment of target genomic regions using gene-specific primers. Enrichment in the methylated DNA precipitation sample was evaluated relative to the immunoglobulin G (sample), using input DNA as a positive control for PCR amplification.

qRT–PCR

qRT–PCR was performed using Power SYBR Green Mastermix (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems 7300 real-time PCR machine as previously described (Yu *et al.*, 2007a). All primers were designed using Primer 3 and synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA) and are listed in Supplementary Table S3. All PCRs were performed in triplicate.

Tissue microarray analysis

Tissue microarray analysis was done in the University of Michigan Prostate Cancer Specialized Program of Research Excellence Tissue Core (SPORE), with informed consent of the patients and prior institutional review board approval.

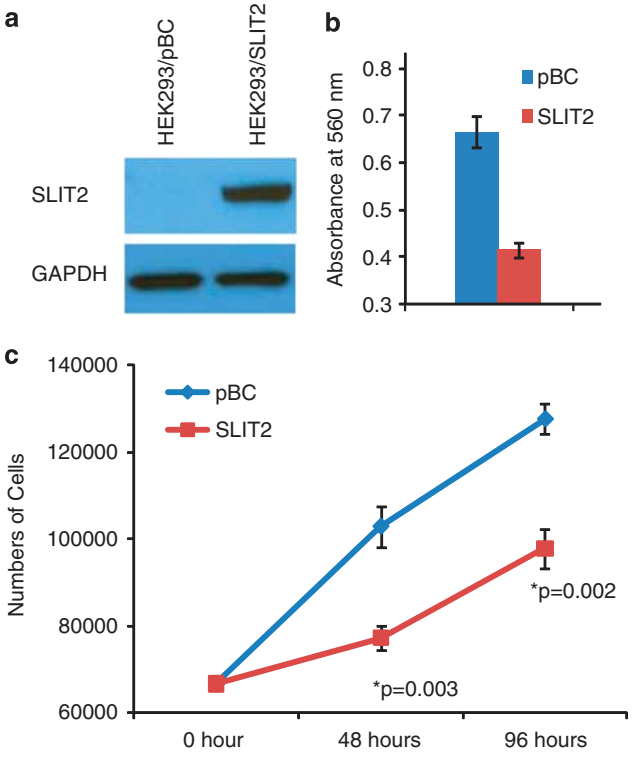

Figure 7 SLIT2 inhibits prostate cancer cell invasion and proliferation. (**a**) Immunoblot analysis of SLIT2 in HEK293/SLIT2 stable cells and the control HEK293/pBC cells. (**b**) SLIT2 overexpression inhibits LNCaP cell invasion. LNCaP cells were incubated with a conditioned medium taken from HEK293/SLIT2 or HEK293/pBC control cell for 48 h and subjected to Boyden Chamber Invasion assay. (**c**) SLIT2 overexpression reduces LNCaP cell proliferation. Cell proliferation assay was performed in LNCaP cells incubated in conditioned medium taken from HEK293/SLIT2 or HEK293/pBC for 48 and 96 h, respectively.

The tissue microarray contained 360 cores taken from 105 patients. Out of these, 169 cores taken from 79 patients were evaluable, the remainder being either lost or purely stromal (191). Standard biotin–avidin complex immunohistochemistry was performed using an SLIT2 antibody (HPA023088, Sigma) and the staining was scored as previously described (Yu *et al*., 2007a). Briefly, product score was used as an overall measure of staining percentage and intensity. Specifically, intensity was coded by an integer ranging from 1 to 4 (none = 1, weak = 2, moderate = 3 and strong = 4) and this number was multiplied by staining percentage to produce the product score for each core. These core-level product scores were then aggregated into a patient-level product score by taking the median score over the set of tumor cores for each patient as the measure of SLIT2 expression for each patient/tissue type combination.

RNA-Seq analysis
Total RNA was isolated from prostate tissues and prepared into libraries for next-generation sequencing according to the manufacturer's protocol (Illumina, San Diego, CA, USA). Massively parallel sequencing was performed using the Genome Analyzer (Illumina) and the sequence was aligned to the reference genome using the Illumina pipeline.

Microarray data analysis
All microarray expression studies and corresponding patient information were extracted from the ONCOMINE database. Log2 transformed intensities of *SLIT2* and *EZH2* genes were then mean-centered across the samples per gene per study and a linear model was fitted to estimate the association significance. For Kaplan–Meier survival analysis, patient samples in each study with SLIT2 expression values greater than its median intensity were grouped as 'SLIT2+', others were grouped as 'SLIT2−'. Survival analysis was then performed in R 2.9 (http://www.r-project.org) and *P*-value was reported by log-rank test.

Cell invasion and proliferation assays
Cell invasion assay was performed with Boyden Chamber (Corning, Corning, NY, USA) coated with Matrigel (BD Biosciences) as previously described (Yu *et al*., 2007a). Briefly, LNCaP cells were harvested and resuspended in RPMI 1640 medium without serum but with HEK293/pBC- or HEK293/SLIT2-conditioned medium at a density of 10^6 cells/ml. The lower chamber of the transwell was filled with 600 µl RPMI 1640 medium with 10% fetal bovine serum and with HEK293/pBC- or HEK293/SLIT2-conditioned medium. LNCaP cell suspension was applied onto the matrigel membrane, and incubated at 37 °C for 24–48 h. Cells migrated through membrane were stained with crystal violet, and further dissolved in acetic acid and read at an absorbance of 560 nm. Cell proliferation assay was done as previously described (Yu *et al*., 2007a).

Conflict of interest

AMC is a cofounder of Compendia Biosciences. Other authors declare no potential conflict of interest.

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