Polycomb protein EZH2 regulates tumor invasion via the transcriptional repression of the metastasis suppressor RKIP in breast and prostate cancer

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Running title: Epigenetic silencing of RKIP expression in cancer metastasis

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Abbreviations used:
FBS: fetal bovine serum; GAPDH: glyceraldehydes-3-phosphate dehydrogenase; HRP: horseradish peroxidase; NF-kB: nuclear factor-kappa B; PBS: Phosphate Buffer Saline; SAHA: suberoylanilide hydroxamic acid.

Keywords: metastasis; EZH2; Raf-1 kinase inhibitor protein (RKIP); methylation; epigenetics
Abstract

Epigenetic modifications such as histone methylation play an important role in human cancer metastasis. EZH2, which encodes the histone methyltransferase component of the polycomb repressive complex 2 (PRC2), is overexpressed widely in breast and prostate cancers and epigenetically silences tumor suppressor genes. Expression levels of the novel tumor and metastasis suppressor RKIP have been shown to correlate negatively with those of EZH2 in breast and prostate cell lines as well as in clinical cancer tissues. Here we show that the RKIP/EZH2 ratio significantly decreases with the severity of disease and is negatively associated with relapse-free survival in breast cancer. Using a combination of loss- and gain-of-function approaches, we found that EZH2 negatively regulated RKIP transcription through repression-associated histone modifications. Direct recruitment of EZH2 and Suz12 to the proximal E-boxes of the RKIP promoter was accompanied by H3-K27-me3 and H3-K9-me3 modifications. The repressing activity of EZH2 on RKIP expression was dependent on HDAC promoter recruitment and was negatively regulated upstream by miR-101. Together, our findings indicate that EZH2 accelerates cancer cell invasion, in part, via RKIP inhibition. The data also implicate EZH2 in the regulation of RKIP transcription, suggesting a potential mechanism by which EZH2 promotes tumor progression and metastasis.
Introduction

Cancer cells are characterized by an unbalanced and dramatically altered epigenetic state compared to normal counterparts. Histone modifications and DNA methylation are among the most studied epigenetic mechanisms that control the accessibility of target gene promoters to positive or negative transcriptional signals and regulate gene expression. Epigenetic alterations related to transcriptional inactivation of tumor suppressor genes form part of a regulatory mechanism that determines the initiation, maintenance and progression of malignant cell transformation during cancer development (1, 2). Recent findings have linked the inactivation of the target promoters in tumors with aberrant expression and activity of proteins that participate in the repressing systems that catalyze the epigenetic change.

Histone alterations involve the trimethylation of histone 3 at lysine 27 or 9 (H3-K27-me3 or H3-K9-me3) that are generally associated with transcriptional repression, whereas di-or tri-methylation of H3 at lysine 4 (H3-K4-me2/me3) and acetylation of H3 at lysine 9 (H3-K9-ace) are associated with transcriptional activation (1, 3). The essential epigenetic systems involved in heritable repression of gene activity are the polycomb repressing complexes PRC1 and PRC2, which consist of the polycomb group (PcG) proteins, the DNA methyltransferases (DNMTs) and the histone deacetylases (HDACs) (4). Physical and functional links among the 3 epigenetic silencing systems have been described in both normal and cancer cells (5, 6). PRC2 is believed to initiate gene silencing by inducing H3-K27 trimethylation, while PRC1 maintains the repressive chromatin structure through mono-ubiquitination of histone H2A at lysine 119 (H2A-K119) (7). H3-K27 trimethylation is catalyzed by the SET domain of the histone methyltransferase PcG protein, enhancer of zeste homolog 2 (EZH2) (8). EZH2 catalytic activity requires the presence of 3 additional PcG proteins, namely, suppressor of zeste 12 (Suz12), embryonic ectoderm development (eed) and retinoblastoma binding proteins 4 or 7 [RbBP4 (RbAP48) or RbBP7]. These 4 proteins constitute the core components of PRC2. For methylation-dependent gene silencing, PRC2 is thought to be the first complex recruited to DNA through the involvement of various intermediate molecules such as the long non-coding RNA hox transcript antisense RNA (HOTAIR), resulting in EZH2-mediated H3-K27-me3 (9). This mark serves as an anchorage point for the further recruitment of PRC1, DNMTs and HDACs, which contribute to chromatin compaction and transcriptional repression (10).
Although polycomb-mediated H3-K27-me3 has been shown to pre-mark genes for de novo DNA methylation in cancer (11), gene silencing by H3-K27 trimethylation independent of promoter DNA methylation is also very frequent in cancer cells (12). This finding supports the significance of expression and activity of H3-K27-me3-inducing EZH2 protein in human malignancies (10). Overexpression of EZH2 was first linked to cancer by microarray studies of breast and prostate cancer (13, 14). Experimental support for the oncogenic action of EZH2 has been provided by induction of anchorage-independent colony growth and promotion of invasion in vitro by overexpression of EZH2 in the breast epithelial cell line H16N2 (14). In addition, down regulation of EZH2 expression by siRNA has been shown to decrease the proliferation of prostate cancer cells in vitro (13).

Different mechanisms have been described to explain the overexpression and increased activity of EZH2 in various cancer models. Among them, an acquired missense mutation (Y641) within the SET domain of EZH2 associated with enhanced catalytic efficiency for H3-K27 trimethylation has been described in B-cell lymphomas (15), whereas deletions of microRNA-101, a negative upstream regulator of EZH2 expression, have been reported in prostate cancer (16).

The link between EZH2 overexpression and tumor invasiveness/metastasis is supported by the suppressing function of EZH2 on the expression of several microRNAs that regulate the expression of PRC1 proteins (17), Kruppel-like factor 2 (18), and the epithelial to mesenchymal transition (EMT)-suppressor gene CDH1 that encodes the E-cadherin protein (19). EZH2 activity has been reported to be required for repression of the CDH1 transcription by the EMT-inducer transcription factor Snail (20, 21). CDH1 shares common expression patterns and regulatory mechanisms with another metastasis and EMT-suppressor gene product, namely Raf-1 kinase inhibitor protein (RKIP) (22). RKIP was initially identified as a potent inhibitor of Raf-1/MEK/ERK, NF-κB, and G-protein-coupled receptor signaling pathways (23-25). RKIP has been further identified as a metastasis suppressor in prostate cancer since its loss of expression in prostate cancer cells conferred a metastatic phenotype, whereas restoration of its expression in a xenograft murine model diminished metastasis (26). Besides prostate cancer, depletion of RKIP expression and activity has now been reported in distant metastases of various cancer types,
including breast, gastric, colorectal and hepatocellular carcinomas, and its expression has been proposed as a prognostic marker for disease-free survival in patients diagnosed with the above cancers. Similar to CDH1, the loss of RKIP expression has been associated with EMT induction, enhanced angiogenesis and vascular invasion, as well as with protection against drug-, radio- and/or immuno-mediated apoptosis during anti-tumor therapy. Although the critical role of RKIP in tumor progression has been documented, its transcriptional regulation still remains largely unclear. We have previously reported that RKIP expression, similar to CDH1, is directly repressed by the EMT-inducer Snail in prostate and breast cancer, however, no other direct or indirect regulators of RKIP transcription have been identified.

In the present study we question the direct involvement of EZH2 in RKIP transcriptional repression in prostate and breast cancer models as well as whether EZH2 association with increased tumor invasiveness might be attributed to RKIP repression. Based on the reported low RKIP levels and the elevated EZH2 expression in invasive prostate and breast carcinomas and metastases, we hypothesized that EZH2 may repress RKIP transcription by inducing histone H3-K27 trimethylation and that the RKIP inhibition is closely related to EZH2-mediated effects on promoting tumor invasion. Our findings support our hypothesis and demonstrate that EZH2 accelerates cell invasion, at least in part, via transcriptional repression of the metastasis suppressor RKIP. We further show that the repressive activity of EZH2 on RKIP transcription required the presence of the EMT-inducer Snail.
Materials and methods

Cell lines and reagents

The prostate carcinoma cell lines DU-145, PC-3 (both metastatic bone-derived human androgen-independent prostatic adenocarcinomas) and LNCaP (non-metastatic bone-derived human androgen-dependent prostatic adenocarcinoma) as well as the breast cancer cell lines T47D, MCF7 and MDA-MB231 were obtained from the American Type Culture Collection (ATCC). The cell lines were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) as described previously (32). The transformed prostate epithelial cell line PrEC-LSHAR expressing SV40 large and small antigen, hTERT and androgen receptor was obtained from Dr. William Hahn (Harvard Medical School) and was cultured as previously described (27). The HDAC inhibitor SAHA was purchased from Santa Cruz Biotechnology Inc., (Santa Cruz, CA) and diluted in DMSO. The anti-α tubulin monoclonal antibody (Clone B-5-1-2) (T-5168) was obtained from Sigma (St. Louis, MO). The antibodies for E-Cadherin (4065), EZH2 (4905), and Suz12 (3737) were purchased from Cell Signaling Technology (Danvers, MA). The antibodies for EED (clone H-300) (Sc-28701), and RbAp48 (clone K-15) (Sc-12434) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-Bmi1 (clone F6) and rabbit anti-RKIP (N-term) (36-0700) antibodies were obtained from Upstate (Lake Placid, NY) and Invitrogen (Carlsbad, CA), respectively. The antibodies used for the ChIP assays, were as follows: anti-EZH2 and control IgG (Millipore, Billerica, MA); rabbit monoclonal anti-SUZ12 (3737S) (Cell Signaling, Danvers, MA); polyclonal anti-H3-K9-me3 (39161), anti-H3-K4-me3 (39159), anti-H4-Ac (39179) and anti-H3-K27-me3 (39157) (Active Motif, Carlsbad, CA). The small interfering RNA (siRNA) against Snail and scrambled siRNA control used for transient cell transfections were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, CA). For all transient transfections, Lipofectamine2000 (Invitrogen, Carlsbad, CA) was used as a transfection reagent.

Plasmid constructs and retroviral vectors

The wild type and E-box-mutant RKIP promoter (2.2Kb) activities were determined by using the luciferase reporter plasmid constructs RKIP-Luc w/t and RKIP-Luc mut, respectively (28). For the transient ectopic expression of EZH2, EZH2 mutant in SET domain (EZH2 H689A), eed and Suz12 we used expression plasmids containing the full-length cDNA of the corresponding genes under the control of a CMV
promoter, as described previously (29). The relevant empty vector was used as a negative control (EVC). The retroviral expression vectors for HOTAIR, miR-101 and miR-145 were kind gifts of Drs. Howard Chang (Stanford University, CA) (14) and Yin-Yuan Mo (Southern Illinois University, IL), respectively. The mammalian expression vectors for wild type and mutant EZH2 as well as SUZ12 were kindly provided by Dr. Danny Reinberg (New York University, NY). The retroviral repressing vectors siSUZ12, siEZH2 or sigfp were kindly provided by Drs. Yi Zhang and Yue Xiong (30), respectively (both from University of North Carolina, Chapel Hill, NC). The expression vectors for RKIP were described previously (31).

Cell extracts and Western Blot analysis
Cells extracts were prepared and Western blotting was carried out as previously described (32).

Quantitative real time RT-PCR
Total cellular RNA was extracted with the Trizol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed using random hexamer primers (Applied Biosystems, Foster City, CA). The resulting cDNAs were used for real-time RT-PCR using SYBR-Green Master PCR mix (QIAGEN, Valencia, CA) in triplicates. Sample’s running and data collection were performed on ABI7500 (Applied Biosystems, Foster City, CA). β-actin, GAPDH and HPRT were used as an internal standard. The specific primers used were as follows:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>H-SUZ12</td>
<td>AAA CGA AAT CGT GAG GAT GG</td>
<td>CCA TTT CCT GCA TGG CTA CT</td>
</tr>
<tr>
<td>H-BMi1</td>
<td>GTC CAA GTT CAC AAG ACC AGA CC</td>
<td>ACA GTC ATT GCT GCT GGG CAT CG</td>
</tr>
<tr>
<td>H-EED</td>
<td>ATT GTG TGC GAT GGT TAG GC</td>
<td>TGT CGA ATA GCA GCA CCA CA</td>
</tr>
<tr>
<td>h-RbAP48</td>
<td>TGA CCA TAC CAT CTG CCT GTG</td>
<td>ACT GCC GTA TGC CCT GT AAG</td>
</tr>
<tr>
<td>hECad</td>
<td>TGC CCA GAA AAT GAA AAA GG</td>
<td>GTG TAT GTG GCA ATG CGT TC</td>
</tr>
</tbody>
</table>
The primer sequences for EZH2 and RKIP have been previously described (19, 33)

**Transient reporter and expression assays**

To determine the effect of PRC2 PcG proteins on RKIP promoter activity, exponentially grown LNCaP cells in 48 well plates were transiently co-transfected with 0.2 ug/well wild type or E-box-mutant RKIP-Luc reporter plasmids and 0.05ug/well EZH2, EZH2 H869A, SUZ12, EED or EVC expression vectors using Lipofectamine2000. The transfection was performed for 24h and according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). Where appropriate, 6h post-transfection the cells were treated with 200nM of the HDAC inhibitor SAHA and cultured up to 24h. Luciferase activity in protein extracts was measured 24h post-transfection in an analytical luminescence counter (Promega, Madison, WI). Data were normalized to protein concentration levels using the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

**Transient cell transfection with small interfering RNA (siRNA)**

LNCaP cells were plated in a 48-well plate in an antibiotic-free growth medium supplemented with 10% FBS and cultured until confluence reached 80-90%. Twenty pmoles of Snail siRNA or a relevant amount of control scrabbled siRNA (siCON) was mixed with Lipofectamin2000 in reduced serum medium Opti-MeM (Gibco, Invitrogen Corporation, NY). Transfection was performed for 48 h according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). Where needed, 48 h post-transfection the cells were co-transfected in the same medium with RKIP-Luc w/t reporter construct and EZH2 expression vector for 24 h as described above. All the combinations with the empty control vector (ECV) were included. Luciferase activity was measured as described above. Snail inhibition was confirmed at the protein level using western blot analysis.

**Retroviral infection**

To generate retroviruses, all retroviral expression vectors with the exception of pLZRS-hotair were transfected into packaging cell 293-GP2 (Clontech) as previously described. Phoenix ampho packaging cells (Orbigen) were used for retroviral vector pLZRS-hotair. pLZRS-hotair transfected Phoenix cells were
selected with puromycin (1.5ug/ml) for 4 days. Cells were changed to regular medium once after selection, viruses were harvested after 24 and 48 hours.

**CHiP assay**

Briefly the cells were grown to near 70% confluence and cross-linked by adding 37% formaldehyde to a final concentration of 1% and incubated at 37°C for 15 min. Cross-linking was stopped by addition of glycine to a final concentration of 0.125 M. The cells were washed twice with ice-cold PBS and pelleted in PBS containing protease inhibitors. The nuclei were isolated in a buffer containing 50mM HEPES-KOH, pH 7.5, 140mM NaCl, 1mM EDTA, 10% Glycerol, 0.5% NP40, 0.25% Triton-X and protease inhibitors. The nuclei were then subjected to sonication for a total of 4 mins at 70% amplitude using a Cole Palmer Ultrasonic processor in the following buffer 10mmM Tris-HCl, pH8.0, 200mM NaCl, 9mM EDTA, 0.5mM EGTA, 01% Na-Deoxycholate, 0.5% N-laurylsarcosine and 0.1% Triton X-100. The resulting chromatin was centrifuged for 15 mins and quantified. 80-100ug of chromatin was pre-cleared for 3 h at 4°C with 50% slurry of protein A, or G, beads in Tris-EDTA (TE) (depending on the isotype of the antibody used) in the presence of 20 μg of salmon sperm DNA and 1 mg of bovine serum albumin/ml. After incubation, the beads were pelleted, and the supernatant was immuno-precipitated with antibodies of interest at 4°C overnight. The immune complexes were collected with Protein A/G agarose beads as prepared for pre-clearing for 4 hours at 4.C. The bead-antibody complex was then washed 5x with RIPA wash buffer (50mM HEPES, 500mM LiCl, 0.1mM EDTA, 1.0% NP-40 and .7% Na-Deoxycholate) and once with TE containing 50mM NaCl. The immune complexes were eluted with 50mM Tris-Hcl pH8.0, 10mM EDTA and 1% SDS. Elution was carried out at 65°C for 30mins followed by reversal of cross-links at 65.c overnight. The DNA was subjected to Rnase A treatment at 37.C for 2 hours, and purified by Proteinase K digestion at 55.C for 2 hours followed by phenol-chloroform extraction and ethanol precipitation. The purified DNA was dissolved in 50 μl Tris-EDTA, and 2 μl was used for PCR.

The primers used for CHiP were as follows:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>RKIP-F</td>
<td>CCA AAA CCC AAA CAT TTC TCA</td>
</tr>
<tr>
<td>RKIP-R</td>
<td>CCT TGC TTT TCT CCT GCA CT</td>
</tr>
<tr>
<td>Myt1-F</td>
<td>ACA AAG GCA GAT ACC CAA CG</td>
</tr>
<tr>
<td>Myt1-R</td>
<td>GCA GTT TCA AAA AGC CAT CC</td>
</tr>
</tbody>
</table>
GAPDH-F: TAC TAG CGG TTT TAC GGG CG
GAPDH-R: TCG AAC AGG AGG AGC AGA GAG CGA

Invasion assays

Invasion assays were carried out in 24-well TC plates with transwell inserts. The polycarbonate membrane (8-um pore size) of transwell fluoroblok inserts (Costar) was coated with 90ul of the diluted (1:25 in serum-free medium) matrigel (BD). Chemoattractive medium (600 ul medium with 10% FBS) was added to the lower chambers of the 24-well plates. Cells were serum starved for 6 hrs and 3x10^4 cells were added into each insert. Cells were incubated at 37°C with 5% CO2 for 20 hrs, stained with fluorescence probe (Molecular Probe) and the number of cells invaded through the membrane were determined from digital images captured on an inverted microscope.

Case selection and tissue microarrays.

A PCa progression tissue microarray (TMA) was constructed from cases of clinically localized PCa obtained from a radical prostatectomy series at the University of Michigan. PCa metastases TMAs were developed from samples obtained through the Rapid Autopsy Program within the Michigan Prostate SPORE Tissue Core (34). The progression TMA 100 consisted of 309 evaluable cores taken from 99 total patients; 92 cores of non-neoplastic prostate (from 39 cases), 23 cores of BPH (from 8 cases), 19 cores of PIN (from 12 cases), 142 cores of localized PCa (from 50 cases), and 33 cores of metastatic, hormone-refractory PCa (from 11 cases) (35). Two PCa autopsy arrays 79A and 79B were constructed from soft tissue and bone metastases taken from 30 available autopsies. TMA 79A consists of 303 evaluable cores of primary PCa and soft tissue metastases of the liver, lung, lymph node, adrenal, bladder, dura, and seminal vesicles. The TMA 79B consisted of 129 evaluable cores included 72 cores (from 17 cases) of bone metastases in addition to primary PCa and soft tissue metastases. All tissue procurement and analysis in this study was approved by the Institutional Review Board. Histological processing of all clinical samples was performed in the University of Michigan Hospital's accredited Pathology Department using a standardized procedure to assure uniform sample preparation for each TMA.
Immunohistochemistry and evaluation.

TMA slides were deparaffinized, rehydrated to water, and antigen retrieved by pretreatment with Citrate Buffer, pH6.0 for 10 minutes with microwaving. After Peroxidase blocking, the slide were incubated with 1:400 dilution of RKIP or EZH2 antibodies on a DAKO AutoStainer using the LSAB+ detection kit and counterstained with Hematoxylin. Staining intensity was scored by a genitourinary pathologist (R.M.) as negative [1], weak [2], moderate [3], or strong [4] based on the amount of stain detected. The percent of positive stained cells was determined by counting 100 cells in 2 random fields. Protein levels in each sample were reported as Expression Index (EI), which is a product of staining intensity and the percentage of positive staining as described previously (36).

Meta-Analysis of Oncomine Database and Survival Analysis

The expression RKIP and EZH2 transcripts in PCa tissues were obtained from meta-analysis of our recently established cancer gene microarray meta-analysis public database (37). Heatmaps were generated using the “heatmap.2” function from the “gtools” package in the R statistical program. Neither a column nor row dendrogram was computed to reorder the values (parameters “Rowv” and “Colv” were set to “NULL” and “dendrogram” was set to “none”). No trace line was drawn (parameter “trace” set to “none”), and the bottom and left margins were set to 12 and 9, respectively (parameter “margins” set to “c(12,9)”). All other parameters used the default settings. The van’t Veer human breast cancer data set consists of 117 breast tumor samples was used for survival analysis as shown in Fig. 1g (38). The association between RKIP/EZH2 expression and relapse-free survival (RFS) within 5 years of diagnosis was analyzed using available outcome data. Samples in the data sets were separated into four groups according to RKIP and EZH2 expression. Survival curves were generated using the “survival” package in R. The p-value was computed using the “survdiff” function in this package, which compares all 4 groups.

Statistical analysis

The Mann-Whitney U and Kruskal-Wallis H tests determined significant differences between values obtained from cells that were treated under different experimental conditions. Analysis of the IHC staining intensity was also performed by using the non-parametric Kruskal-Wallis and Wilcoxon rank tests. The
Pearson correlation coefficient was used to compare the staining intensities of the 2 markers within the samples and tested against a null hypothesis that there is a lack of correlation (correlation coefficient=0). Probability (P) was set significant at the level of 0.05. All statistical analyses were performed using the SPSS or SAS 9.2 software.
Results

**RKIP expression negatively correlates with EZH2 levels in prostate human cancer samples and the RKIP/EZH2 ratio predicts relapse-free breast cancer survival.**

As a first step to determine whether EZH2 plays a role in maintaining the repressed state of RKIP expression, we interrogated publicly available DNA microarray expression datasets, in search of a correlation between RKIP and EZH2 expression in prostate cancer samples. Ten datasets were identified with normal adjacent prostate tissue (NAP), benign prostatic hyperplasia (BPH), localized prostate cancer (PCA), and metastasis of prostate cancer (MPC) (33). Only three of them included both RKIP and EZH2.

As previously reported (13), while the RKIP expression was low, expression of EZH2 was highly upregulated in all three datasets as the cancer progressed (p<0.05 for both EZH2 and RKIP) (Fig. 1a, 1b and supplementary fig. 1a). Importantly, we also observed a strong negative correlation between RKIP expression and EZH2: r = -0.67 (p = 0.0004) across all samples in dataset #1, and -0.65 across PCA and MPC samples (p = 0.009) (Fig. 1c). Similar results were obtained with dataset #2 and 3 (supplementary fig. 1a).

The association between high EZH2/lower RKIP expression and metastasis shown at the mRNA level was further validated at the protein level using prostate tissue microarrays. Human BPH, PCA and MPC samples were stained for RKIP and EZH2 protein expressions using immunohistochemistry. EZH2 staining density was found higher in MPC samples compared to PCA, while the opposite results were observed for RKIP (Fig. 1d). For both EZH2 and RKIP the expression differences among BPH, PCA and MPC samples were statistically significant, thus indicating that among the studied groups the metastatic prostate tumors have the lowest RKIP/EZH2 protein ratios (Fig. 1e). Lastly, the staining densities obtained for both RKIP and EZH2 proteins were found inversely correlated with a Pearson Correlation Coefficient of -0.42 (p<0.0001) (Fig. 1f). On average, the RKIP stain index is 114.9 (95% CI: 98.3 – 131.4) points higher than the EZH2 stain index in a core (p<0.0001).

Since low RKIP expression in primary tumors was also a strong positive predictive factor for prostate and...
breast cancer recurrences (38, 39), we examined the prognostic value of different \textit{EZH2}/\textit{RKIP} expression combinations in breast cancers in which the expression of \textit{EZH2}, \textit{RKIP} and clinical outcome are available in published microarray expression data set (38). We found that high \textit{EZH2} and low \textit{RKIP} expression were associated more significantly with the development of metastasis within 5 years of primary diagnosis than either high \textit{EZH2} or low \textit{RKIP} expression alone (Fig. 1g). These results implicate \textit{EZH2} as another possible repressor of \textit{RKIP} expression in prostate and breast cancers and suggest that \textit{EZH2} and \textit{RKIP} may be in the same regulatory pathway affecting cancer metastasis.

\textit{Inverse correlation between RKIP and EZH2 expression levels in prostate and breast cancer cell lines with different metastatic potential}

To examine the connection between RKIP and \textit{EZH2} in metastasis, we first determined RKIP and \textit{EZH2} expression in cancer cell lines of different metastatic potential. Consistent with the expression patterns that were observed in prostate cancer samples, the expression of \textit{EZH2} was found to be high in the low RKIP-expressing metastatic prostate cancer cell line DU145, while the expression was low in high RKIP-expressing non-metastatic LNCaP cells (Fig. 2a). Similar results were also observed in metastatic (MDA-MB231) and non-metastatic (MCF7 and T47D) breast cancer cell lines (Fig. 2b). In addition to \textit{EZH2} and RKIP, we also examined the expression levels of other components of the polycomb repressing complexes as well as E-cad, a known target of \textit{EZH2} suppression. Besides \textit{EZH2}, eed and Bmi1 (a basic component of PRC1 core) (40) were the only other PcG proteins found significantly elevated in the metastatic compared to non metastatic cell lines and negatively associated with the levels of RKIP and E-cad (Fig. 2a-b). Real time RT-PCR results in the above prostate (Fig. 2c) and breast (Fig. 2d) cancer cell lines were consistent with protein expression levels revealed by WB (Western Blot) analysis with exception of \textit{EZH2} expression in prostate cancer cell lines. Consistent with a previous report (41) in prostate cancer cell line, the expression levels of \textit{EZH2} mRNA were quite similar despite an increased \textit{EZH2} protein expression in the metastatic cell line DU145. Nevertheless, an inverse correlation between RKIP and \textit{EZH2} protein expression was observed in the tested breast and prostate cancer cell lines. The above findings establish a positive association between high \textit{EZH2}/low \textit{RKIP} expression motif and a metastatic cell phenotype and provided us with a cell-based system to investigate the possible
involvement of EZH2 in the mechanism that mediates RKIP repression in breast and prostate malignancies.

**EZH2 is directly involved in the suppression of RKIP expression during breast and prostate cancer progression**

To investigate the regulatory effects of PcG proteins on RKIP expression, we expressed EZH2 or small interference RNA (siRNA) for EZH2 or Suz12 by retroviral infection in both prostate and breast cancer cell lines with different metastatic capacities. Ectopic expression of wild type EZH2 in the non metastatic breast and prostate cell lines MCF7 and LNCaP resulted in a significant decrease of baseline RKIP expression when compared with the empty vector control (EVC) (Fig. 3a). In contrast, infection of MCF7 cells with a SET domain mutated EZH2 (EZH2 H689A)-expressing retrovirus was incapable of reducing RKIP expression levels (Fig. 3a). Since H689A mutation inactivates EZH2’s methyltransferase activity towards H3K27 our findings suggested that EZH2 enzymatic activity was essential for RKIP repression. This methyltransferase dependent effect of EZH2 on RKIP expression was also observed in the transformed LSHAR prostate epithelial cell line (Fig. 3a). The inhibitory role of EZH2 on RKIP promoter transcriptional activity was further shown by real-time RT-PCR in LNCaP, MCF7, and LSHAR cells, where the relative RKIP mRNA expression was found significantly reduced only in cells expressing the wild type (Fig. 3b). To further examine the causal role of EZH2 on RKIP repression, we silenced the EZH2 expression in the metastatic DU145 and MDA-MB231 cell lines using small interfering RNA (siRNA) and monitored the expression of RKIP. siEZH2-treated cells had significantly higher RKIP protein levels compared to the sigfp control as shown in Figure 3c. Likewise, the relative RKIP mRNA expression was also significantly increased in siEZH2 expressing DU145 and MDA-MB231 cells (Fig. 3d). In contrast, silencing of Suz12 by siRNA in DU145 did not increase RKIP protein expression (Fig. 3e).

microRNA-101 (miR-101) expression decreases during prostate and breast cancer progression and this abnormal miR-101 downregulation has been proposed as causative factor of EZH2 overexpression in the above tumors (42). Consistent with the notion that EZH2 regulates RKIP expression, ectopic expression of miR-101 in DU145 caused a robust induction of RKIP protein as detected by Western blot (Fig. 3f). As expected, expression of miR-101 decreased EZH2 protein expression (Fig. 3f). The effect
was specific for miR-101 since expression of another microRNA, miR-145, had no effect on the expression levels of EZH2 or RKIP (Fig. 3f). The above findings therefore suggest a direct suppressive role of EZH2 on RKIP expression during breast and prostate cancer initiation and progression.

**RKIP transcription is repressed via direct recruitment of the EZH2-containing PRC2 complex to the proximal E-boxes of the RKIP promoter**

Conceptually, EZH2 could regulate RKIP expression by either one of two distinct mechanisms. It may act directly on the RKIP promoter in a cis-binding site-dependent manner. Alternatively, EZH2 can affect the stability of RKIP mRNA. To differentiate between these two models we performed luciferase reporter assays in LNCaP cells using a plasmid carrying a 2.2 kb RKIP promoter (2.2Kb) and EZH2 expression vectors. Ectopic expression of wild type EZH2 resulted in significant inhibition of RKIP promoter activity (p=0.013) while expression of the mutant EZH2 H689A had little effect (p=0.068) (Fig. 4a). Similar results on RKIP promoter activity were observed after ectopic expression of other PRC2 PcG proteins Suz12 and eed in LNCaP cells (Fig. 4b). Next, we determined whether EZH2 represses RKIP expression by physically associating with the RKIP promoter by a ChIP assay with EZH2 specific antibodies in DU145 cells. We found that EZH2 is directly recruited to the proximal regions of the RKIP promoter and this recruitment is associated with the detection of high levels of the repressive marks H3-K27-me3 (Histone 3-Lys 4-methylation) and H3-K9-me3 (Fig.4c). In addition to the repressive marks, we also detected the activation-related histone modifications, H3-K4-me2 and H4-Ac (H4-acetylation) marks on the RKIP promoter (Fig. 4c). As expected, the RKIP promoter was also found enriched with Suz12, the other core component of the PRC2 complex. These findings reveal a direct association of RKIP transcriptional repression in metastatic prostate cancer with both the recruitment of PRC2 and EZH2-mediated H3-K27 trimethylation of the RKIP promoter. PRC2 represses transcription initiation partly by recruiting HDACs to the promoter (11). To assess the necessity of HDACs as part of the repressive complex that catalyzes the transcriptional repression of RKIP, we monitored the RKIP promoter activity in LNCaP cells transfected with the wild type EZH2 expression vector in the absence or presence of the HDAC inhibitor SAHA. Cell treatment with SAHA reversed EZH2-mediated repression of the RKIP promoter activity and restored
**RKIP** expression in LNCaP cells (Fig. 4d), indicating that HDAC activities act complementary in EZH2-mediated **RKIP** suppression.

**Snail but not HOTAIR is involved in the EZH2-mediated repression of RKIP promoter**

Since none of the PRC2 subunits are sequence specific DNA-binding proteins, it is not precisely known how EZH2 histone methyltransferase is recruited to target mammalian genes. Among the suggested recruiters is a long non-coding RNA, known as HOTAIR, which was initially implicated in PRC2 recruitment to the human HOXD cluster (43). During breast cancer progression, expression of HOTAIR becomes dysregulated and overexpressed. It has been demonstrated that dysregulated HOTAIR expression induced genome-wide re-targeting of PRC2 and promote breast cancer metastasis (44). It is possible that PRC2 is re-targeted to the **RKIP** promoter leading to its subsequent repression during breast cancer progression. To test this possibility, we ectopically expressed HOTAIR in the breast cancer cell line MDA-MB231 (Fig 5a) and monitored the expression levels of **RKIP** (Fig 5b-c). Overexpression of HOTAIR increased the expression of **ABL2**, a positive regulator of cancer metastasis as demonstrated previously (43) but had no detectable effect on **RKIP** mRNA (Fig. 5b) or protein expression (Fig. 5c) levels. This finding suggests that HOTAIR is not involved in **RKIP** suppression via EZH2.

An alternative explanation for PRC2 recruitment to the **RKIP** promoter is via transcription factors with direct DNA-binding sites on the target promoter. The only transcription factor that has been reported so far to regulate **RKIP** transcription is Snail (33). Snail binds directly to E-boxes of **RKIP** promoter and facilitates its suppression (33). Since EZH2 bound to a region of the **RKIP** promoter that contains three E boxes, it was possible that Snail is involved in EZH2-mediated **RKIP** suppression. To test this possibility, we generated an **RKIP** reporter plasmid with mutated E-boxes that prevent efficient Snail binding (28). Expression of EZH2 in LNCaP cells did not have a significant effect on the transfected mutated E-boxes **RKIP** reporter, indicating that EZH2 suppressive activity on the **RKIP** promoter depends on Snail binding to the **RKIP** promoter (Fig. 5d). To further examine the direct involvement of Snail in EZH2-mediated **RKIP** suppression, we silenced the expression of Snail in LNCaP cells by Snail siRNA and monitored the **RKIP** promoter activity in the presence or absence of EZH2 expression (Fig. 5e). Snail silencing resulted in reversal of EZH2-mediated inhibition of **RKIP** promoter activity after ectopic expression of EZH2.
(p=0.061) and significantly reduced the enrichment of *RKIP* promoter in EZH2 molecules as shown by ChIP analysis on the target promoter (Fig. 5f). The observed differences were not due to differences in levels of Snail expression because expression or knocking down of EZH2 in prostate cancer cells did not affect levels of Snail expression (Fig. 5g). However, despite repeated attempts we failed to detect the co-targeting of both Snail and EZH2 to the same region in *RKIP* promoter by sequential CHIP assays (data not shown). Overall, these findings demonstrate that Snail is required for EZH2-mediated repression of the *RKIP* promoter and link the inhibition of *RKIP* expression with the combined suppressive activities of PRC2 and Snail on the target promoter.

**EZH2 accelerates cancer cell invasion by inhibiting RKIP expression**

It has been reported that downregulation of EZH2 inhibited cancer cell anchorage-independent growth, proliferation, and invasion (45). Since decreased EZH2 expression also caused an increase in *RKIP* expression (Fig. 3c), it was possible that gain of *RKIP* expression is the cause of the observed effects due to the loss of EZH2 expression in cancer cells. To address this possibility we designed an experimental approach whereby we tested whether the decrease of proliferation, anchorage-independent growth or invasion by silencing of EZH2 in DU145 and MDA-MB231 cell lines could be reversed by ectopic silencing of *RKIP* expression. While the blockage of *RKIP* expression had no observable effect on anchorage-independent growth or proliferation (supplementary Fig. 1b-c), it effectively reversed the decrease in invasiveness due to the loss of EZH2 (Fig. 6a-e). It has been shown that RKIP inhibits cell invasion by repressing the expression of *MMP* (matrix metalloprotease) genes in breast cancer cells (31, 46). Consistently, we observed a good correlation between the *MMP13* expression and invasion in MDA-MB231 cells with altered expression of *EZH2* or/and *RKIP* (Fig. 6c, left panel). Concomitantly, when the prostate epithelial cell line PrEC LSHAR was infected with EZH2-expression retrovirus it acquired increased invasive properties, which were significantly diminished back to the baseline invasion rate after expressing RKIP (Fig. 6f-g). Altogether, these findings suggest that RKIP inhibition by EZH2 might be part of the molecular mechanism by which EZH2 promotes invasion and metastasis in prostate and breast malignancies.
Discussion

RKIP is a proven metastasis suppressor of prostate and breast cancer. Consistent with its anti-metastatic effect, RKIP expression is inversely correlated with tumor aggressiveness and is almost lost in metastatic tumors thus predicting a poor prognosis (22). The molecular mechanisms by which RKIP expression is downregulated during cancer progression and metastasis have not been completely elucidated. Epigenetic silencing by histone methylation has also been shown to play an important role in human cancer metastasis. The histone lysine methyltransferase EZH2 is overexpressed in cancers and is associated with cancer aggressiveness with poor prognosis. In cancer cells, deregulated EZH2 activity represses normal expression of tumor suppressor or metastasis genes. In breast and prostate cancers, among the genes found to be directly targeted and silenced by EZH2 are metastasis suppressor genes CDH1 (19), FOXC1 (47) and DAB21P (48). In the present study we identify RKIP as an additional transcriptional target of EZH2 and mediator of EZH2 pro-metastatic effects.

We observed a significant positive association between high EZH2/low RKIP expression ratio and tumor aggressiveness/metastasis in cell lines and human cancer samples. Functionally we showed that the high EZH2/low RKIP ratio is a strong positive predictive factor for breast cancer recurrence and poor survival. Thus, our results provide for the first time evidence of the involvement of EZH2 in the mechanism that mediates RKIP repression during cancer progression. The recapitulation of EZH2 and RKIP expression levels in cancer cell lines provided us with a genetically tractable system to investigate the causal role of EZH2 in silencing RKIP expression in cancer metastases. Significant inhibition of RKIP expression was observed in low EZH2-expressing cancer cells when expression of EZH2 was restored, whereas EZH2 silencing resulted in recovery of the lost RKIP expression in the high EZH2-expressing metastatic cell lines. Expression of EZH2 in a transformed prostate epithelial cell line with defined genetic alterations was also capable of suppressing RKIP expression, suggesting that abnormally elevated EZH2 levels in normal prostate epithelia may contribute to initiation of tumorigenesis via repressing, among other genes, the tumor suppressor RKIP. Suppression of RKIP expression by EZH2 requires its histone lysine methyltransferase catalytic activity implying that the EZH2-mediated regulation of RKIP expression is at the level of transcription initiation.
Three lines of evidence indicate that EZH2 regulates RKIP expression at the level of transcription. Firstly, genetic manipulation of EZH2 expression in cancer cell lines resulted in the change of RKIP mRNA and protein expression levels. Secondly, the RKIP promoter activity was found significantly diminished after overexpression of the wild type EZH2 but not the SET-mutant EZH2 vectors, indicating that histone modifications related to EZH2-mediated H3-K27-me3 might take place on the RKIP promoter for RKIP suppression. Lastly, we demonstrated the physical association of EZH2 and Suz12 with the RKIP promoter in intact cancer cells.

EZH2 is part of a multi-component protein complex named PRC2. The other components include Suz12, eed and RbAp48. Consistent with previous findings in human cancer samples (13, 14), only the expression of the EZH2 subunit is elevated in both breast and prostate metastatic cell lines. In normal cells the histone methyltransferase activity of EZH2 requires its binding to Suz12 and eed. Unlike EZH2, silencing of Suz12 by siRNA in high EZH2-expressing DU145 cells did not reveal any significant RKIP induction. On the contrary, in low EZH2-expressing LNCaP cells ectopic expression of Suz12 or eed alone was sufficient to repress RKIP expression. These findings, therefore, suggest that EZH2 catalytic activity may not require the presence of Suz12 and eed when overexpressed in cancer. However, in low EZH2 expressing cancer cells overexpression of other subunits is enough to shore up the activity of the PRC2 complex. In contrast to Suz12, the expression levels of the upstream regulator of EZH2 expression, miR-101, was shown to interfere negatively with EZH2-mediated RKIP suppression. miR-101 has been shown to directly repress EZH2 and abnormal miR-101 downregulation has been described in breast and prostate cancer lines (42), suggesting that it might be the cause of EZH2 overexpression and RKIP inhibition. The reversal of EZH2-mediated RKIP suppression by ectopic expression of miR-101 supports the above hypothesis and identifies miR-101 as one of the positive regulators of RKIP expression through EZH2 inhibition.

Consistent with the detection of bound EZH2 in the RKIP promoter, we found the RKIP promoter marked with H3-K27 trimethylation (me3). In addition to H3-K27-me3, we also detected the presence of H3-K9-me3 repressing marks on the RKIP promoter in DU145 cells, which indicates that in addition to the
EZH2 methyltransferase activity other histone methyltransferases also play a role in RKIP suppression. Unexpectedly, activation-related histone modifications H3-K4-me2 and H4-Ac were also detected on the RKIP promoter. Co-localization of activating and repressing marks has been detected in a vast number of gene promoters in the human genome and the gene expression levels was shown to be correlated with both the absolute and relative levels of the activating H3-K4-me3/H4-Ac and the repressive H3-K27-me3/H3-K9-me3 modifications (9). The expression of RKIP is low in cancer cells. However, its expression is not completely shut-off and can be reactivated. Our data, therefore, suggest that these histone modifications together may define the chromatin dynamics important for RKIP repression and derepression in response to different stimuli.

Another important component for EZH2-mediated RKIP suppression was shown to be the presence of HDAC, since cell treatment with the HDAC inhibitor SAHA abolished the suppressive activity of EZH2 on the RKIP promoter. This finding suggested that either SAHA relieves EZH2 catalytic activity on the RKIP promoter, or EZH2-mediated H3K27me3 of RKIP promoter might not be sufficient for promoter silencing and other repressing enzymes including HDACs are necessary to be recruited and interact with the initial PRC2 complex for further chromatin compaction and transcriptional repression of RKIP. This is consistent with the fact that H3K27me3 mark usually serves as an anchorage point for the further recruitment of additional repressive elements on the target promoters such as PRC1, DNMTs and HDACs (49). Accordingly, we believe that SAHA more likely obstructs the preservation of the suppressive mark on the RKIP promoter rather than the initial EZH2-mediated H3-K27 trimethylation of the RKIP promoter.

To delineate how EZH2 is recruited to the RKIP promoter we examined the involvement of the known PRC2 recruiter HOTAIR. The absence of changes in RKIP expression after ectopic expression of HOTAIR argues that HOTAIR might not participate in the recruitment process. Contrarily, silencing of Snail, a transcription factor with high expression levels in metastatic breast and prostate tumors and a direct repression on the RKIP promoter (33), resulted in inhibition of RKIP promoter enrichment in EZH2 molecules and reversal of EZH2-mediated repression of RKIP promoter activity. EZH2 recruitment was dependent on the efficient binding of Snail on the E-boxes present on the RKIP promoter, since mutations
in E-boxes abolished the suppressive effect of EZH2 on the \textit{RKIP} promoter activity. Thus, our work identifies Snail as one of the possible recruiters of EZH2 to RKIP promoter and links the reduction of RKIP expression in metastatic prostate and breast malignancies with the combined suppressive activities of PRC2, HDAC and Snail on the target promoter. However, these findings motivate further studies to test if our previously shown Snail-mediated RKIP suppression requires the presence of EZH2-triggered histone modifications. Furthermore, at present it is not clear if Snail plays a direct role in tethering EZH2 to the \textit{RKIP} promoter.

\textbf{RKIP inhibits cancer metastasis at least via inhibition of EMT-associated cancer cell invasion and the corresponding mesenchymal cell phenotype (50). On the other hand, abnormally elevated EZH2 levels promotes cell invasion leading to cancer metastasis. We showed that by genetically manipulating the expression levels of \textit{RKIP} we were able to reverse the EZH2-mediated cancer cell invasive phenotype. This observation demonstrates one potential mechanism by which EZH2 accelerates cancer cell invasion and metastasis through the inhibition of \textit{RKIP} expression. Additional studies in \textit{in vivo} metastatic models are needed to validate the dependence of EZH2 pro-metastatic activity on RKIP suppression.}

\textbf{In conclusion, our findings provide evidence that histone modifications, regulated by PcG proteins, and HDACs, are involved in the transcriptional repression of the metastasis suppressor gene \textit{RKIP}, thereby permitting tumor cell expansion. Collectively, these studies confer new data on the molecular mechanism by which EZH2 promotes cancer progression and aggressiveness in breast and prostate malignancies and identifies novel targets for therapeutic intervention.}
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgements

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47. Du J, Li L, Ou Z, et al. FOXC1, a target of polycomb, inhibits metastasis of breast cancer cells.


Figure legends

Figure 1

Increasing EZH2 followed by decreasing RKIP levels are associated with a progressive cancer disease and are hallmarks for metastasis and poor survival. a) Heat map of RKIP, EZH2, Snail and E-cad expression profiles obtained by a representative publicly available DNA microarray expression dataset. The database consisted of 52 human prostate samples of different histopathology grouped as: NAP: normal adjacent prostate tissue, BPH: benign prostate hyperplasia, PCA: prostate cancer adenocarcinoma, and MPC: metastatic prostate carcinoma. Rows correspond to individual genes and columns represent individual patients. Color density is arranged in order from greatest decrease in expression at left (red) to greatest increase at right (light yellow). EZH2 and Snail mRNA are strongly expressed in MPC, in contrast to RKIP and E-cad. b) Box plots presenting the mean ± SEM values of EZH2 and RKIP mRNA expression for each of the indicated groups in the studied DNA microarray database. EZH2 expression is significantly increasing with the severity of the disease (p<0.05), while RKIP is significantly decreasing (p<0.05). The statistical significance of the expression differences among the various histological groups was determined using pairwise comparisons. c) EZH2 mRNA expression is inversely correlated with RKIP expression in the studied prostate samples (p=0.0004). The plot scale represents expression level after logarithmic transformation (log_2 value) of the expression ratios for both RKIP and EZH2 (r=-0.67) d) Representative staining of EZH2 and RKIP protein expression in prostate cancer adenocarcinoma (PCA) and prostate metastatic cancer (PMC) tissue arrays, as assessed by immunohistochemistry. A clear increase in RKIP staining accompanied by decreased RKIP staining is observed in PMC compared to PCA. e) Box plots presenting the mean and quartile values of EZH2 and RKIP protein expression in tissue arrays for each of the studied groups. EZH2 protein expression is significantly increasing in metastatic samples (p<0.0001), accompanied by significant decrease in RKIP expression (p<0.0001). The statistical significance of the expression differences among the various histological groups was determined using Kruskal-Wallis test. f) EZH2 staining density expression is inversely correlated with that of RKIP in the studied samples (Pearson Correlation Coefficient = -0.42 and p<0.0001). g) High EZH2 and low RKIP mRNA levels are associated with breast cancer metastasis and bad prognosis. Kaplan Meier curve assessing the disease free survival of 97 breast cancer patients.
based on RKIP and EZH2 mRNA levels obtained by a publicly available DNA microarray expression dataset (38). A statistically significant increase in relapse free survival was observed in patients with high RKIP/EZH2 expression ratios (p=0.0294).

Figure 2

Increasing EZH2 expression levels accompany reduced RKIP expression in metastatic breast and prostate cell lines. a) Representative western blot analysis of EZH2, SUZ12, EED, BimI1, RKIP and E-cadherin in metastatic (DU145, PC3) and non-metastatic (LNCaP) prostate cancer cell lines. Tubulin expression was served as a loading control. b) Protein expression of EZH2, Suz12, eed, RbAP48, BimI1, RKIP and E-cadherin protein expression in metastatic (MDA-MB231) and non-metastatic (T47D and MCF7) breast cancer cell lines, as assessed by western blot. c) Relative mRNA levels of PCR2 subunits EZH2, Suz12, eed, RbAP48 and the metastasis suppressors RKIP and CDH1, as determined by real-time RT-PCR in LNCaP, PC3 and DU145 cell lines. Actin mRNA level was used as internal control. d) Relative mRNA expression of EZH2 and RKIP in MDA-MB231, T47D and MCF7 cell lines. All statistical analyses were performed using unpaired two-tailed t-test.

Figure 3

EZH2 is directly involved in RKIP repression during prostate and breast cancer progression. a) Representative western blot analysis of EZH2 and RKIP expression in protein lysates derived from the non-metastatic LNCaP and MCF7 cells and the prostate epithelial cell line PrEC LSHAR cell lines expressing the wild type or the EZH2 H689A mutant proteins. EVC: Empty vector control. Tubulin expression was used as a loading control. Relative levels of EZH2/tubulin or RKIP/tubulin are indicated. b) Relative RKIP mRNA levels assessed by real-time RT-PCR in LNCaP, MCF7 cells or PrEC expressing the wild type or H689A mutant EZH2 proteins. Actin mRNA level was used as internal control. c) Protein expression of RKIP and EZH2 in the metastatic DU145 and MDA-MB231 cell lines expressing siRNA against EZH2 or sigfp. Relative levels of EZH2/tubulin or RKIP/tubulin are indicated. d) Relative RKIP mRNA expression in DU145 or MDA-MB231 cells expressing siEZH2 or sigfp control as assessed by real-time RT-PCR. e) Protein expressions of RKIP and EZH2 in DU145 cell expressing silencing Suz12 or
gfp control siRNAs as assessed by western blot. f) EZH2-mediated RKIP suppression is negatively regulated by miR-101. Downregulation of EZH2 expression by ectopic expression of its suppressor miR-101 upregulates RKIP expression as assessed by western blot. miR-145 overexpression was used as negative control.

**Figure 4**

Direct recruitment of EZH2-containing PRC2 complex on RKIP promoter promotes H3-K27 and H3-K9 trimethylation and RKIP transcriptional inactivation. a) Ectopic expression of wild type EZH2 suppresses RKIP promoter activity in LNCaP cells. LNCaP cells were transfected with an RKIP-Luciferase reporter plasmid simultaneously with an empty vector control (EVC) or wild type EZH2 (EZH2), or mutant EZH2 (H869A) expression vectors for 24h. At the end of the transfection time cells were lysed and luciferase expression was assessed using a luminometer and expressed as relative luciferase units (RLU). The RKIP reporter vector used carried the full RKIP promoter with approximate size 2.2 KDa. b) Ectopic expression of PRC2 components EED and SUZ12 suppresses RKIP promoter activity in LNCaP cells. c) ChIP analysis on DU145 cells showing the association of EZH2 and SUZ12 with the proximal E-boxes of RKIP promoter accompanied by an increase in H3 methylation. The putative E-boxes in RKIP promoter are shown as red boxes. The locations of primers used for ChIP assays are marked by black arrows (amplicons). ChIP analysis was performed using specific antibodies that recognize EZH2, SUZ12, repressing marks (H3-K27-me3 and H3-K9-me3), and activation marks (H3-K4-me2 and H4-Ac), as well as control IgG. GAPDH promoter was used as a reference active control, whereas Myt-1 (an inhibitor of cdc2/cyclin B–dependent initiation of mitosis) as an EZH2-suppressed control promoter. d) HDAC activities are required for EZH2-mediated RKIP suppression. The HDAC inhibitor SAHA inhibits EZH2-mediated inhibition of RKIP promoter activity. LNCaP cells were first transfected with the RKIP-Luc reporter simultaneously with the EZH2 expression vector or EVC for 24h followed by treatment with 200nM SAHA for another 24h. e) LNCaP treatment with 200nM SAHA after cell transfection with EZH2 or empty vectors reverses the EZH2-mediated inhibition of RKIP protein expression. Beta-actin was used as a loading control.
Snail and not HOTAIR is involved in EZH2 recruitment to RKIP promoter. a) Overexpression of HOTAIR in MBA-MD231 cells infected with HOTAIR expressing retrovirus or EVC as assessed by Real-time PCR. b). qRT-PCR analysis of ABL2 and RKIP mRNA expression in HOTAIR or EVC expressing MBA-MD231 cells. The expression of the indicated genes was normalized to the expression level of GAPDH (n=3). c) Western blot examines expression of EZH2 and RKIP protein in HOTAIR expressing MDA-MB231 cells. d) RKIP suppression by EZH2 is dependent on Snail binding to proximal E-boxes of RKIP promoter. LNCaP cells were co-transfected with an RKIP-Luc vector carrying mutated E-boxes and EZH2 expression vector or empty control vector (EVC). Twenty-four hours after transfection, cells were harvested for luciferase assay. Relative luciferase units (RLU) were determined using a luminometer. e) Snail silencing in LNCaP cells by Snail siRNA causes a marginally significant reversal of EZH2-mediated inhibition of basal RKIP promoter activity. LNCaP cells were initially transfected with Snail siRNA or control siRNA for 48h followed by co-transfection with wild type RKIP-Luc plasmid and EZH2 expression vector or empty vector control (EVC) for additional 24 hours. Transfected cells were harvested for luciferase assay as described in fig. 5e and the expressions of Snail and EZH2 were assayed by Western blotting (upper panel). f) Expression of Snail is required for the binding of EZH2 to the RKIP promoter. Chromatin immunoprecipitations (ChIPs) were performed in control or snail knockdown DU145 cells using antibodies to RKIP or control IgG. RKIP promoter was amplified with primers that span the putative Snail-binding site and quantified by real-time PCR. GAPDH was used as a negative control. These results are an average of two independent experiments performed in triplicate. g) Western blotting showing protein expressions of Snail and EZH2 in DU145 and LNCaP cell lines expressing siRNA against EZH2 and EZH2 cDNA, respectively. Relative levels of EZH2/tubulin or RKIP/tubulin are indicated.

EZH2 accelerates cancer cell invasion by inhibiting RKIP expression. a) Inhibition of invasion by siEZH2 is reversed by silencing of RKIP. DU145 metastatic prostate cancer cells were infected with
siEZH2 or siRKIP or the combination retroviruses. The number of invaded cells was counted 24 hours later using a matrigel-based invasion assay. These results are an average of two independent experiments performed in triplicate. Small-interference RNAs against gfp and luciferase (Luc) were used as negative controls. b) The protein expression of EZH2 and RKIP in each indicated infected cell was assessed by western blot. Tubulin was served as loading control. c) Breast cancer cells MDA-MB-231 infected with the indicated retroviruses. Number of invaded cells was counted as described in (a). Left panel- relative mRNA level of MMP-13 as determined by real-time RT-PCR in different MDA-MB231 cells as indicated. Actin mRNA level was used as internal control. d) The levels of RKIP and EZH2 protein expressions in each indicated infected cell were monitored by western blot. e) A representative field of matrigel membrane from c) with the invaded cells stained in green. f) EZH2-mediated invasion is abolished in presence of high levels of RKIP. Prostate epithelial cells (PrEC) LSHAR were transfected with EZH2 or RKIP expression vectors or the combination of both and the number of invaded cells was counted as described above. Empty vector and gfp were used as negative expression controls. g) The protein levels of RKIP and EZH2 in each indicated infected cell were assessed by western blot.
Ren et al. Fig 3

**a)**

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**f)**

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a) RKIP2.2

- LUC

- EVC
- EZH2
- EZH2 H689A

p = 0.013
p = 0.068

b) RLU/10^5

- EVC
- EZH2
- Suz12

p = 0.009
p = 0.032

d) RLU/10^5

carrier control
SAHA 200nM
p = 0.035

EVC
EZH2
EZH2

SAHA 200nM

fold enrichment

- Myt
- RKIP

- EZH2 Ab
- control IgG
- H3-K27-Me3 Ab
- H3-K9-Me3 Ab
- Suz12 Ab
- H3-K4-Me Ab
- H4-Ac Ab

GAPDH

0
10^2
10^3
10^4

b) a)

p = 0.009
p = 0.032

p = 0.035

p = 0.013

p = 0.068

p = 0.009

p = 0.032

p = 0.035

p = 0.013

p = 0.068

p = 0.009

p = 0.032

p = 0.035

p = 0.013

p = 0.068

b) a)
DU145

- siLuc, sigfp
- siEZH2, siLuc
- siEZH2, siRKIP
- siRKIP, sigfp

# of cells invaded

- p = 0.0076
- p = 0.00186

MDA-MB231

- siLuc, sigfp
- siLuc, siEZH2
- siRKIP, sigfp
- siRKIP, siEZH2

# of cells invaded

- p = 0.0013
- p = 0.0022

MMP-13

- p = 0.016
- p = 0.027

PrEC

- LSHAR

- EZH2
- tubulin
- RKIP

f)

gfp, EVC
- gfp, EZH2
- RKIP, EVC
- RKIP, EZH2

# of cells invaded

- p = 0.0026
- p = 0.0027

f)

- EZH2
- tubulin
- RKIP

e)
Polycomb protein EZH2 regulates tumor invasion via the transcriptional repression of the metastasis suppressor RKIP in breast and prostate cancer

Gang Ren, Stavroula Baritaki, Himangi Marathe, et al.

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