Published in final edited form as:

Nat Cell Biol. 2010 November ; 12(11): 1108–1114. doi:10.1038/ncb2116.

Cyclin-dependent kinases regulate epigenetic gene silencing through phosphorylation of EZH2

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Abstract

The Polycomb group (PcG) protein, enhancer of zeste homologue 2 (EZH2), has an essential role in promoting histone H3 lysine 27 trimethylation (H3K27me3) and epigenetic gene silencing^{1–4}. This function of EZH2 is important for cell proliferation and inhibition of cell differentiation, and is implicated in cancer progression^{5–10}. Here, we demonstrate that under physiological conditions, cyclin-dependent kinase 1 (CDK1) and cyclin-dependent kinase 2 (CDK2) phosphorylate EZH2 at Thr 350 in an evolutionarily conserved motif. Phosphorylation of Thr 350 is important for recruitment of EZH2 and maintenance of H3K27me3 levels at EZH2-target loci. Blockage of Thr 350 phosphorylation not only diminishes the global effect of EZH2 on gene silencing, it also mitigates EZH2-mediated cell proliferation and migration. These results demonstrate that CDK-mediated phosphorylation is a key mechanism governing EZH2 function and that there is a link between the cell-cycle machinery and epigenetic gene silencing.

PcG proteins are important regulators of epigenetic gene silencing^{8–10} and have key roles in developmental patterning, X-chromosome inactivation and stem cell maintenance^{5, 6, 11}. Many of the proteins in this family function in two distinct protein complexes termed Polycomb-repressive complex 1 (PRC1) and Polycomb-repressive complex 2 (PRC2). PRC2 contains four core subunits of EZH2, EED, SUZ12 and RbAp 48(46) in humans or E(z), esc, Su(z)12 and Nurf55 in flies^{1–4}. EZH2 is the catalytic subunit of PRC2 and

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AUTHOR CONTRIBUTIONS

S.C. performed most of the experiments and analysis. L.R.B. generated mutation constructs. A.N.R. performed PRC2 complex purification and *in vitro* HMTase assays. Y.P. performed immunofluorescent chemistry. L.G. provided technical assistance. X.Z. and A.B. provided reagents and technical advices. H.H. conceived the study. S.C., J.A.S. and H.H. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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Note: Supplementary Information is available on the Nature Cell Biology website

contains a SET domain responsible for H3K27me³^{1–4}. This chromatin mark is commonly associated with silencing of differentiation genes in organisms ranging from plants and flies to humans^{8–10}, suggesting that EZH2 is a master suppressor of cell differentiation.

Many studies also link EZH2 to oncogenesis^{7, 12}. Compared with corresponding normal tissues, EZH2 levels are frequently elevated in numerous human cancers, including prostate cancer⁷. The abundance of EZH2 correlates with advanced tumour stage and poor prognosis for the patient⁷ and forced expression of EZH2 promotes cancer cell proliferation and migration. Conversely, knockdown of EZH2 by RNA interference inhibits cancer cell proliferation and migration^{7, 13}. The role of EZH2 in tumorigenesis may reflect its activity in silencing of tumour suppressor genes, such as *p16^{INK4A}*, *ADRB2* and *DAB2IP*^{14–16}.

Few studies have been performed to understand how the function of this regulatory protein is itself controlled. *EZH2* gene transcription is negatively regulated by the tumour suppressor protein, RB, and the microRNA, *miR-101* (refs 13 and 17). Akt phosphorylates EZH2 at Ser 21 and inhibits its methyltransferase activity¹⁸. However, it is unclear how the function of EZH2 is positively regulated, and maintained, in proliferative cells.

EZH2 expression and activity are higher in proliferating, rather than fully differentiated, cells and tissues^{17,19,20}. Accordingly, EZH2 has a crucial role in the maintenance of stem cell pluripotency and suppression of cell differentiation^{6,11,21}. As EZH2 commonly functions in highly proliferative cells that have high CDK activities, we hypothesized that EZH2 might functionally interact with CDKs in proliferative cells. Indeed, EZH2 harbours one perfectly matched (Thr 350) and two imperfectly matched (Thr 421 and Thr 492) CDK phosphorylation motifs (K(R)S(T) PXX(R), where X is any residue²²; Supplementary Information, Fig. S1a). To assess phosphorylation by CDKs, GST fusions of the amino terminus (amino-acid residues 1–559) and carboxy terminus (amino-acid residues 560–741) of EZH2 were used in *in vitro* protein-kinase assays. The EZH2 N-terminal fragment was phosphorylated by the CDK1–cyclin B1 complex, but the C-terminal fragment was not (Fig. 1a). As expected, histone H1B, a known CDK1 substrate, was readily phosphorylated in these assays, whereas no phosphorylation of the control glutathione S-transferase (GST) protein was observed (Fig. 1a). Mutation of Thr 350 to alanine (T350A) resulted in approximately 60% reduction in phosphorylation of the N-terminal EZH2 fragment mediated by CDK1 (Fig. 1b). In contrast, approximately 30% or no reduction in phosphorylation was observed when T421A and T492A mutants were used as substrates (Fig. 1b). This suggests that Thr 350 in EZH2 is the major site phosphorylated by the CDK1–cyclin B1 complex *in vitro*. Further analysis showed that CDK2–cyclin E and CDK2–cyclin A, but not CDK6–cyclin D1, can also phosphorylate EZH2, and that this phosphorylation is largely or completely abolished by the T350A mutation (Fig. 1c). These data indicate that the EZH2 protein can be specifically phosphorylated at the Thr 350 residue by different CDKs *in vitro*. Notably, this residue is present in a consensus CDK phosphorylation motif that is evolutionarily conserved from fruit flies to humans (Fig. 1d; although the putative CDK site in the fruitfly homologue of EZH2 is imperfectly matched with the CDK consensus motif there is a similar motif in the mitosis regulatory protein, nucleophosmin (NPM or B23) that has been shown to be phosphorylated by CDK1; ref. 30).

To determine whether CDK1 and CDK2 can phosphorylate EZH2 at Thr 350 *in vivo*, an antibody specific to phosphorylated Thr 350 (anti-Thr 350-P) was raised and purified. The antibody reacted with wild-type but not EZH2^{T350A} in both 293T (Fig. 2a) and prostate cancer LNCaP cells (Supplementary Information, Fig. S1b). This reaction was blocked by a peptide containing the phosphorylated Thr 350, but not by the corresponding nonphosphorylated peptide (Supplementary Information, Fig. S1c). Treatment of cellular proteins with λ protein phosphatase completely abolished the reaction of this antibody with

EZH2 (Fig. 2b), confirming that the anti-Thr 350-P antibody is specific to phosphorylated Thr 350. Ectopic expression of CDK1–cyclin B1 or CDK2–cyclin E substantially increased Thr 350 phosphorylation of both endogenous and exogenous wild-type EZH2, but not EZH2^{T350A}, in LNCaP cells (Fig. 2c and Supplementary Information, Fig. S1b). Thr 350 phosphorylation of EZH2 was inhibited in cells overexpressing the CDK inhibitors, p21^{WAF1} (Fig. 2c) and p27^{KIP1} (Supplementary Information, Fig. S1b). Thr 350 phosphorylation of endogenous EZH2 was substantially reduced by knockdown of endogenous CDK1 and CDK2, and this effect was enhanced by further treatment with the CDK inhibitor, roscovitine (Fig. 2d). Thr 350 phosphorylation of both endogenous and ectopically expressed EZH2 in 293T cells was confirmed by mass spectrometry analysis (Fig. 2e and Supplementary Information, Fig. S1d and S1e). Furthermore, Thr 350-phosphorylated EZH2 was invariably co-localized with the proliferation marker Ki-67 in human prostate tumours (Fig. 2f). We also found that CDK1 and CDK2 interact with EZH2 *in vitro* and *in vivo* (Supplementary Information, Fig. S2). These data indicate that CDKs can phosphorylate EZH2 at Thr 350 under various physiological and pathological conditions.

The biological function of EZH2 is primarily reflected by its global repression of gene transcription^{7,11}. Thus, we performed microarray analysis to gain molecular insights into the effect of EZH2 Thr 350 phosphorylation on gene expression in mammalian cells. Endogenous EZH2 was knocked down by an *EZH2*-specific siRNA (*EZH2* siRNA), or restored to physiological levels by ectopically expressing siRNA-resistant wild-type EZH2 (EZH2^{SR}) or a siRNA-resistant EZH2^{T350A} mutant (T350A^{SR}) in LNCaP cells (Supplementary Information, Fig. S3a and S3b). mRNA samples were then collected for oligonucleotide microarray profiling analysis. For comparison, microarray analysis was performed in LNCaP cells treated with the CDK inhibitor, roscovitine. Additionally, it has been shown previously that histone deacetylase (HDAC) proteins can physically interact with the PRC2 complex²³, and treatment of cells with the HDAC inhibitor trichostatin A (TSA) blocks EZH2-mediated gene silencing^{7,23}. Therefore, as a positive control, we also performed microarray analysis of LNCaP cells treated with TSA. As demonstrated in Figure 3a (lanes 2 and 3), a large set of genes were transcriptionally derepressed by EZH2 knockdown and repressed again in cells with the restored expression of wild-type EZH2. Consistent with the role of HDACs in concert with the PRC2 complex^{7,23}, inhibition of HDACs by TSA also resulted in derepression of this set of EZH2 target-genes (Fig. 3a, lane 7). Most importantly, a great percentage (> 78%) of EZH2-target-genes failed to be repressed by expression of the siRNA-resistant EZH2^{T350A} mutant (Fig. 3a, lane 4). Similarly, we detected that more than 74% of EZH2-repressed genes are not repressed when EZH2^{T350A} is expressed in normal human BJ fibroblasts (Supplementary Information, Fig. S3c, d). Intriguingly, the majority (> 60%) of Thr 350 phosphorylation-regulated EZH2-target-genes were also affected by roscovitine treatment in LNCaP cells (Fig. 3a; lanes 4 and 6, and Supplementary Information, Fig. S3e), although, as expected, roscovitine treatment resulted in a much broader impact on gene expression (Supplementary Information, Fig. S3e). We conclude that CDK-induced Thr 350 phosphorylation of EZH2 is important for its genome-wide repression of gene transcription.

The *HOXA9* gene is a well-studied EZH2 repression target^{1,18,24}. To determine whether EZH2 phosphorylation at Thr 350 affects *HOXA9* expression, endogenous EZH2 was knocked down or restored by ectopic expression of siRNA-resistant wild-type EZH2 or EZH2^{T350A} using the strategy shown in Figure 3a and Supplementary Information, Figure S3b. As expected, knockdown of endogenous EZH2 resulted in an increase in *HOXA9* expression in LNCaP cells (Fig. 3b, left). *HOXA9* expression was repressed again by restored expression of wild-type EZH2. However, this effect was substantially compromised by the expression of EZH2^{T350A} (Fig. 3b, left). Overexpression of CDK2–cyclin E and

CDK1–cyclin B1 also repressed *HOXA9* gene expression (Fig. 3c). This effect was abrogated by EZH2 knockdown (Fig. 3c). Moreover, silencing of endogenous CDK1 and CDK2 increased expression of *HOXA9* (Fig. 3d). No additive effect on *HOXA9* expression was observed in cells where CDK1, CDK2 and EZH2 were knocked down (Fig. 3d). Thus, these data suggest that CDK-mediated Thr 350 phosphorylation on EZH2 is important for its regulation of *HOXA9* expression.

Consistent with the fact that EZH2 is a strong promoter of cell proliferation and migration and a master repressor of cell differentiation^{7,11,17,21,25,26}, our microarray analysis revealed that many genes important for cell growth and differentiation are affected by EZH2 Thr 350 phosphorylation. Knockdown of EZH2 increased *DAB2IP* expression in LNCaP cells, consistent with previous reports that the putative tumour suppressor gene *DAB2IP* (which has a role in cancer cell proliferation and metastasis) is a EZH2 target^{14,27} (Fig. 3b, right). This increase was diminished by restored expression of wild-type EZH2 but not the EZH2^{T350A} mutant (Fig. 3b, right). In addition to *HOXA9*, many other key developmental regulators, including transcription factors in the HOX, FOX and SOX families, are known targets of PRC2¹¹. Our microarray data demonstrated that Thr 350 phosphorylation is important for EZH2-mediated repression of many of these genes (Supplementary Information, Fig. S3f and S3g). These data indicate that Thr 350 phosphorylation of EZH2 is important for its repression of genes either mediating differentiation or blocking cell proliferation and migration.

EZH2-promoted gene silencing is mediated primarily by its function in catalysing H3K27me3 in the promoters of its target-genes^{1,18,24}. Consistently, chromatin immunoprecipitation (ChIP) analysis in LNCaP cells demonstrated that knockdown of EZH2 decreased the level of H3K27me3 in the promoters of *HOXA9* and *DAB2IP* (Fig. 4a, b). This effect was largely reversed by restored expression of wild-type EZH2, but not the EZH2^{T350A} mutant (Fig. 4a, b). Next, we assessed whether Thr 350 phosphorylation directly affects the enzymatic activity of EZH2. *In vitro* histone methyltransferase (HMTase) assays were performed using PRC2 complexes that were either immunoprecipitated from mammalian cells or reconstituted from proteins isolated after baculovirus-mediated expression in insect Sf9 cells. Surprisingly, no difference in HMTase activity was detected *in vitro* between wild-type EZH2 and the EZH2^{T350A} mutant (Supplementary Information, Fig. S4a and S4b). Furthermore, CDK-mediated phosphorylation of EZH2 did not alter core PRC2 complex formation in mammalian or insect cells (Supplementary Information, Fig. S4a and S4b), or the half-life of the EZH2 protein as assessed in LNCaP cells (Supplementary Information, Fig. S4c and S4d). Thus, the impact of EZH2 Thr 350 phosphorylation on H3K27me3 levels in target-gene promoters (Fig. 4b) cannot be attributed to changes in stability, formation or intrinsic HMTase activity of PRC2. We performed ChIP assays to test for changes in PRC2 targeting. Indeed, the binding of EZH2^{T350A} to the promoters of *HOXA9* and *DAB2IP* was much lower, compared with wild-type EZH2 (Fig. 4c, d). These data suggest that EZH2 Thr 350 phosphorylation may affect PRC2 recruitment to its target loci in cells.

Previous studies demonstrated that EZH2 is frequently overexpressed in advanced human prostate cancers, and that ectopic expression of EZH2 promotes proliferation of immortalized RWPE-1 prostate epithelial cells and PC-3 prostate cancer cells⁷, two cell-lines that express relatively low levels of endogenous EZH2 (Supplementary Information, Fig. S5a). Consistent with those studies, ectopic expression of wild-type EZH2 markedly augmented growth of RWPE-1 cells (Fig. 5a). However, EZH2-stimulated proliferation of RWPE-1 cells was largely attenuated by the T350A mutation (Fig. 5a). This attenuation was not because of differences between levels of the wild-type and mutated EZH2 proteins (Fig. 5a, inset). A similar result was obtained in PC-3 cells (Supplementary Information, Fig.

S5b). Consistent with these observations, we demonstrated using soft-agar assay that ectopic expression of wild-type EZH2 markedly enhanced anchorage-independent growth of 22Rv1 prostate cancer cells (Fig. 5b). However, this effect was largely diminished in cells infected with lentiviruses expressing the EZH2^{T350A} mutant (Fig. 5b), although wild-type and mutated EZH2 proteins were expressed at comparable levels (Supplementary Information, Fig. S5c). In addition to cell proliferation, EZH2 also promotes cell migration^{13,28}. Thus, we performed wound healing assays to determine whether Thr 350 phosphorylation affects the role of EZH2 in cell migration. Similarly to the previous report¹³, expression of wild-type EZH2 significantly accelerated migration of RWPE-1 cells (Fig. 5c, d). However, the T350A mutation largely diminished EZH2-promoted migration in this cell line (Fig. 5c, d). Thus, Thr 350 phosphorylation contributes to the tumour-promoting functions of EZH2, including proliferation and migration.

Our data demonstrate that CDKs function as important positive regulators of EZH2 through phosphorylation at the Thr 350 residue. Notably, the motif containing Thr 350 is evolutionarily conserved, suggesting that this regulatory mechanism could be functional in other organisms. Although the T350A mutation does not alter the intrinsic HMTase activity of PRC2 as assessed by *in vitro* assays using HeLa polynucleosomes as a substrate, Thr 350 phosphorylation not only affects H3K27me3 levels in the EZH2 target loci examined, it also regulates the global effect of EZH2 on gene silencing in different cell types. Consistent with these observations, ablation of Thr 350 phosphorylation diminishes the binding of EZH2 to its target loci in cells. Thus, our data identify CDK1- and CDK2-mediated Thr 350 phosphorylation as an important mechanism in control of EZH2-mediated epigenetic gene silencing in mammalian cells.

The function of EZH2 is essential for silencing of differentiation factors, thereby making key contributions to maintenance of stem cell pluripotency^{6,11,21}. We demonstrate that CDK phosphorylation is important for EZH2-mediated silencing of developmental regulators, such as members of the HOX, FOX and SOX families (Fig. 3 and Supplementary Information, Fig. S3f, g) that drive cell differentiation. Thus, CDK phosphorylation may augment the role of EZH2 in inhibiting these transcription factors and reinforce continued proliferation over differentiation. On cell cycle exit at certain stages of development, CDK stimulation of EZH2 would probably decline, which might facilitate desilencing of EZH2 targets and cell differentiation.

In addition to its role in repression of cell differentiation, EZH2 is also important for oncogenesis by regulating cancer cell proliferation and migration^{7,15,17}. We provide evidence that Thr 350 phosphorylation is essential for these functions of EZH2 in prostate cancer cells. Because CDK activity is often elevated in human cancers²⁹, our data suggest that aberrant activation of CDKs may contribute to the aggressive phenotype of tumours by phosphorylating and maintaining the oncogenic and gene-silencing functions of EZH2. This regulatory node may serve as a viable therapeutic target to switch off the tumour-promoting functions of EZH2 in human cancers.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturecellbiology/>

Supplementary Material

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Acknowledgments

We would like to thank M. –C. Hung, Y. Zhang, H. Piwnica-Worms and L. Naldini for plasmids, R. A. Weinberg for BJ cells, K. Zhang for helping in the analysis of mass spectrometry data, and Z. Zhang for critical comments and suggestions. This work was supported in part by grants from the National Institutes of Health (CA134514 and CA130908 to H.H. and GM49850 to J.S.), the Department of Defense (W81XWH-07-1-0137 and W81XWH-09-1-622 to H.H. and W81XWH-07-1-0373 to J.S.), and a Brainstorm Award from University of Minnesota Masonic Cancer Center (to H.H.).

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Figure 1.

CDK1 and CDK2 phosphorylate EZH2 at Thr 350 *in vitro*. **(a)** Left: *in vitro* kinase assay. Recombinant CDK1–cyclin B1 protein complex was incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and the indicated substrates. Reaction samples were resolved by SDS–PAGE and autoradiography. Right: protein substrates indicated by Coomassie blue staining. **(b)** Left: *in vitro* CDK1 kinase assay using a wild-type (WT) EZH2 GST-fusion protein fragment (amino-acid residues 1–559), and T350A, T421A, and T492A mutants of EZH2 (amino-acid residues 1–559), as substrates. Right: protein substrates indicated by Coomassie blue staining. **(c)** Top: *in vitro* CDK2 and CDK6 kinase assays using GST, a wild-type EZH2 GST-fusion protein segment (residues 1–559) and a EZH2 (residues 1–559) T350A mutant, as substrates. Bottom: protein substrates indicated by Coomassie blue staining. **(d)** Comparison of the amino-acid sequence of EZH2 homologues near the CDK phosphorylation site (Thr 350 indicated by asterisk). Uncropped images of blot are shown in Supplementary Information, Fig. S6a.

Figure 2.

CDK1 and CDK2 phosphorylate EZH2 at Thr 350 *in vivo*. **(a)** 293T cells were transfected with plasmids to express Myc-tagged wild-type EZH2 or a EZH2^{T350A} mutant. Ectopically expressed EZH2 proteins were immunoprecipitated with anti-Myc, and resolved by western blot using antibody raised against phosphorylated Thr 350 (anti-T350-P) or anti-Myc. **(b)** EZH2 was immunoprecipitated from 293T cells expressing Myc-EZH2. Immunoprecipitated EZH2 proteins were subjected to λ protein phosphatase treatment and resolved by western-blot analysis with anti-T350-P or anti-Myc antibodies. **(c)** LNCaP cells were transfected with plasmids expressing v5-CDK1 and v5-cyclin B1, v5-CDK2 and v5-cyclin E, or Flag-p21^{WAF1}. Thr 350 phosphorylation of endogenous EZH2 was detected by the anti-T350-P antibody. Immunoblotting of extracellular signal-regulated kinase 2 (Erk2) was included as a loading control. **(d)** LNCaP cells were transfected with siRNAs against *CDK1* and *CDK2* for 48 h and then treated with or without the CDK inhibitor roscovitine, as indicated. Endogenous EZH2 Thr 350 phosphorylation was detected by the anti-T350-P antibody. **(e)** Lysates from 293T cells were immunoprecipitated with the anti-T350-P antibody and resolved by SDS-PAGE gel analysis. The EZH2 band was excised and analysed by LC-MS/MS mass spectrometry. The MS/MS spectrum of the double-charged ion (m/z 382.2) shows that the Thr 350 residue is phosphorylated (low case p in red) in the peptide 348-IKTPPK-353. The b ions (b1–b5) are the fragmentation ions containing the N terminus of the peptide, whereas the y ions (y1–y5) are the fragmentation ions containing the C terminus of the peptide. **(f)** Representative immunofluorescence microscopy images of primary human prostate tumours using anti-T350-P, anti-Ki-67, and DAPI to visualize nuclei ($n = 12$). Scale bar, 10 μm. Uncropped images of blots are shown in Supplementary Information, Fig. S6a.

Figure 3.

The effect of Thr 350 phosphorylation on EZH2-mediated repression of its target-genes. **(a)** Hierarchical clustering of 6,450 genes (represented by 10,276 probe-sets) that exhibited expression differences in LNCaP cells. Lanes 1–4; cells were transfected with *EZH2*-specific siRNA (*EZ4*) and vectors expressing siRNA-resistant *EZH2* or a siRNA-resistant *EZH2*^{T350A} mutant, as indicated. Lanes 5–7; cells were treated with a CDK inhibitor or HDAC inhibitor. Gene profiling data from cells transfected with the indicated siRNA and vectors (lanes 2, 3 and 4) were normalized to that in cells transfected with control siRNA (lane 1), and the data from drug-treatment experiments (lanes 6 and 7) were normalized to vehicle (DMSO) treatment (lane 5). Red and green represent upregulation and downregulation, respectively, as indicated in the scale at the top. Wild-type *EZH2* and *EZH2*^{T350A} mutant proteins were expressed at comparable levels (Supplementary Information, Fig. S3b). Experiments were performed in duplicate ($n = 2$). **(b)** LNCaP cells were transfected with *EZ4* siRNA or plasmids expressing Myc-tagged, siRNA-resistant *EZH2* or Myc-tagged, siRNA-resistant *EZH2*^{T350A}, as indicated (empty vectors were used as a control). At 60 h after transfection, expression of *HOXA9* (left) and *DAB2IP* (right) were analysed by real-time RT-PCR. Asterisks indicate $P < 0.01$. Experiments were performed in triplicate ($n = 3$). **(c)** Prostate cancer cells were transfected with plasmids expressing v5-CDK2 and v5-cyclin E (DU145 cells, left), v5-CDK1 and v5-cyclin B1 (PC-3 cells, right), or an empty control vector, in combination with control or *EZH2* siRNA. At 72 h after transfection, expression of *HOXA9* was evaluated by real-time RT-PCR. Asterisk indicates $P < 0.05$, and double asterisks indicate $P < 0.01$. Experiments were done in triplicate ($n = 3$). Western blots (bottom) were used to identify expression of the indicated proteins. **(d)** DU145 cells were transfected with siRNAs against *CDK1*, *CDK2* and *EZH2* as indicated. At 72 h after transfection, expression of *HOXA9* was evaluated by real-time RT-

PCR. Asterisks indicate $P < 0.01$. Experiments were carried out in triplicate ($n = 3$). Western blots (bottom) were used to identify expression of the indicated proteins. Uncropped images of blots are shown in Supplementary Information, Fig. S6a.

Figure 4.

The effect of Thr 350 phosphorylation on H3K27me3 levels and EZH2 recruitment at EZH2-target-gene promoters. **(a, b)** LNCaP cells were transfected with control or *EZH2*-specific siRNA and plasmids expressing Myc-tagged, siRNA-resistant EZH2 or Myc-tagged, siRNA-resistant EZH2^{T350A} (or empty control plasmids), as indicated. **(a)** At 60 h after transfection, expression of EZH2 was examined by western blot. **(b)** H3K27me3 levels in promoters of the EZH2-target-genes, *HOXA9* (left) and *DAB2IP* (right), were assessed by ChIP assays using anti-H3K27me3 antibodies. Data are means $\pm$ s.d. from three individual experiments ($n = 3$). Asterisks indicate $P < 0.01$. **(c, d)** LNCaP cells were transfected with control or *EZH2*-specific siRNA and plasmids expressing Myc-tagged, siRNA-resistant EZH2 or Myc-tagged, siRNA-resistant EZH2^{T350A} (empty plasmids were used as a control), as indicated. **(c)** At 60 h after transfection, expression of endogenous and restored EZH2 was examined by western blot. **(d)** The binding of Myc-EZH2 and Myc-EZH2^{T350A} to *HOXA9* (left) and *DAB2IP* (right) promoters was examined by ChIP assays with anti-Myc antibody. Data are means $\pm$ s.d. from three individual experiments ($n = 3$). Asterisk indicates $P < 0.05$, double asterisks indicate $P < 0.01$. IgG; immunoglobulin G used as control antibody in ChIP assay. Uncropped images of blots are shown in Supplementary Information, Fig. S6b.

Figure 5.

Phosphorylation of EZH2 Thr 350 is crucial for its function in promoting cell proliferation and migration. **(a)** RWPE-1 cells were transfected with empty control plasmids, or plasmids expressing wild-type EZH2 or the EZH2^{T350A} mutant. Graph shows cell proliferation, as monitored by MTS assay, at the indicated times after transfection. The levels of endogenous and ectopically expressed wild-type and EZH2^{T350A}-mutant proteins were detected with an anti-EZH2 antibody at 48 h after transfection by western blot (inset). Erk2 was included as a loading control. Asterisks indicate $P < 0.01$ when comparing cells transfected with plasmids expressing wild-type EZH2 to those transfected with plasmids expressing EZH2^{T350A}. Data are means $\pm$ s.d. from experiments with six replicates ($n = 6$). **(b)** Effects of EZH2 Thr 350 phosphorylation on anchorage-independent growth of 22Rv1 cells. Representative images of colonies formed by cells infected with lentiviral vectors expressing GFP (control), EZH2 or EZH2^{T350A}, and cultured in medium with agar for two weeks. Scale bar, 300 μ m. Clones with the diameter larger than 300 μ m in ten randomly selected fields were counted (graph, inset). Asterisk indicates $P < 0.01$. Data are means $\pm$ s.d. from three individual experiments ($n = 3$). **(c, d)** Cell migration evaluated by wound healing assay. RWPE-1 cells were transfected with empty control plasmids, or plasmids expressing EZH2 or EZH2^{T350A}. Artificial wounds were created on cells in confluence. Images were taken at 0, 24, 48 and 72 h after wound **(c)**, and the wound widths were measured and quantified **(d)**. Asterisk indicates $P < 0.01$. Data are means $\pm$ s.d. from five individual experiments ($n = 5$). Uncropped images of blot are shown in Supplementary Information, Fig. S6b.