METHODS

Cell lines. Breast cancer cell lines (MCF-7, SKBr3, MDA-MB435, and MDA-MB468); human embryonic kidney cell line (293 and 293T); and human cervical cancer cell line (HeLa) were obtained from American Type Culture Collection (Manassas). The cells were grown on tissue culture dishes in a 1:1 (v/v) mixture of Dulbecco's modified Eagle's minimum essential medium and Ham's F12 medium (DMEM/F12) supplemented with 10% of heat-inactivated fetal bovine serum (FBS). Human MSC cell line was cultured in low glucose DMEM containing 10% FBS. Osteoblast differentiation was induced with low glucose DMEM containing 10% FBS, 10.8 M dexamethasone, 50 μg ml^{-1} ascorbic acid-2 phosphate, and 10 mM β-glycerophosphate. For adipocyte differentiation, hMSCs were cultured in low glucose DMEM with 10% FBS supplemented with 10.7 M dexamethasone, 50 μg ml^{-1} ascorbic acid-2 phosphate, 50 μM indomethacin, 10 μg ml^{-1} insulin, and 0.45 μm 3-isobutyl-1-methylxanthine. The differentiation medium was replaced every 3 days during differentiation period.

Antibodies. Antibodies to c-Myc (1:3,000), haemagglutinin (1:3,000; Roche Molecular Biochemicals), trimethyl-H3K27 (1:10,000), EED (1:3,000; Upstate), SUZ12 (1:2,000), phospho-CDK1 (Thr161; 1:1,000; Cell Signaling), CDK1 (1:1,000), Runx2 (1:1,000), Osteopontin (1:1,000; Santa Cruz Biotechnology) and EZH2 (1:2,000; BD Biosciences) were purchased. Mouse antiserum against the phosphorylation site of EZH2 at Thr 487 were produced with a synthetic phosphopeptide: EDVD(pT)PPRKKKRKH.

CGP74514A treatment. CDK1 inhibitor CGP7451A was obtained from Sigma (Cat # C3353). Cell lines were treated with CGP74514A (2 μM) for 16 h.

Identification of phosphorylation sites by mass spectrometry. To identify *in vivo* phosphorylation site of EZH2, lysates of HeLa cells were immunoprecipitated with anti-EZH2. *In vitro* phosphorylation site of EZH2 was identified by *in vitro* kinase assay using recombinant cyclin B, CDK1 and full-length GST–EZH2. After protein gel electrophoresis, bands were excised from gels, and subjected to tryptic digestion. After being isolated by immobilized metal affinity chromatography, the enriched phosphopeptides were analysed by micro-liquid chromatography/tandem mass spectrometry (LC–MS/MS) by using an Ultimate capillary LC system (LC Packings) coupled to a QSTARXL quadrupole (Q)–time-of-flight (TOF) mass spectrometer (Applied Biosystems). The product ion spectra generated by nanoscale capillary LC-MS/MS were searched against National Center for Biotechnology Information databases for exact matches using the ProID (Applied Biosystems) and MASCOT search programs. Carbamidomethyl cysteine was set as a fixed modification, and serine, threonine and tyrosine phosphorylation were set as variable modifications. All phosphopeptides identified were confirmed by manual interpretation of the spectra.

GST pulldown assays. GST and GST–EZH2 protein (10 μg) were incubated at 4 °C with 2 mg HeLa cell-extract overnight, and the GST-tagged proteins were recovered by incubating the reaction at 4 °C for 3 h with 20 μl glutathione–Sepharose 4B beads. The bead pellet was washed three times with 1 ml buffer (20 mM Tris–HCl at pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100 and 2 mM EDTA). Boiled samples were then subjected to SDS–PAGE.

Plasmids. DNA plasmids encoding CDK1–HA (Addgene plasmid 1888), CDK1-DN–HA (Addgene plasmid 1889), Rc/CMV cyclin B2 (Addgene plasmid 10911) were from Addgene. Dominant-negative mutant CDK1 (DN-CDK1) is a D146N mutant[11]. pcDNA3-His-Myc–EZH2 was a gift from A. Chinnaiyan. Site-directed mutagenesis was performed according to the manufacturer's protocol (Clontech; Palo Alto, CA). Thr 487 in EZH2 was replaced with alanine, using the primer: T487A, 5′-GAGGATGTGGATGCTCCTCCAAGGAAAAAG-3′. To generate constructs for bacterial expression of wild-type and mutant EZH2 tagged with glutathione-*S*-transferase (GST), DNA fragments encoding full-length EZH2 were subcloned into the bacterial expression vector pGEX6P-1 (Amersham Biosciences). Wild-type and mutant EZH2 proteins were inducibly expressed in *Escherichia coli* strain BL21 and purified by glutathione–Sepharose chromatography (Amersham Biosciences).

RNAi plasmid. RNA interference was performed using lentiviral short hairpin RNA (shRNA) libraries[21]. MISSION™ TRC-Hs (Human) shRNA Library were bought from research institute Academia Sinica in Taiwan.

Primers for *CDK1* shRNA were: shRNA-CDK1-A01 (Clone ID TRCN0000000582): 5′-CCGGGCTGTACTTCGTCTTCTAATTCTCGAGAA-TTAGAAGACGAAGTACAGCTTTTT-3′; shRNA-CDK1-B01 (Clone ID TRCN0000000583): 5′-CCGGGTGGAATCTTTACAGGACTATCTCGAG-ATAGTCCTGTAAAGATTCCACTTTTT-3′ and shRNA-CDK1-D01 (Clone ID TRCN0000000585): 5′-CCGGTGGCTTGGATTTGCTCTCGAACTCGAG-TTCGAGAGCAAATCCAAGCCATTTTT-3′.

Quantitative real-time RT–PCR. Total RNA was extracted from cells. Quantitative RT–PCR (qRT–PCR) was performed using SYBR Green dye on a Bio-Rad PCR machine. Briefly, 1 μg of total RNA was reverse transcribed into cDNA using SuperScript III (Invitrogen) in the presence of random hexamers. All reactions were performed in triplicate with SYBR Green Master Mix (Applied Biosystems) plus 1 μM of both the forward and reverse primer according to the manufacturer's recommended thermocycling conditions, and then subjected to melt-curve analysis. The calculated quantity of the target gene for each sample was divided by the average sample quantity of the housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) to obtain the relative gene expression. The primer sequences for the transcript analysed are provided in Supplementary Information, Table S4.

Chromatin immunoprecipitation (ChIP) assays. The ChIP assay was carried out with antibodies against trimethyl-H3K27 (Rabbit polyclonal from Upstate; 07-449), SUZ12 (Rabbit polyclonal from Upstate; 07-379) and C-Myc (Roche Molecular Biochemicals). The assay was performed using the EZ-ChIP kit (Millipore) according to the manufacturer's instructions. The cells were cross-linked for 10 min by addition of formaldehyde to a final concentration of 1%. The cross-linking was stopped by adding 1/20 volume of 2.5 M glycine. This was followed by cell lysis and sonication. Antibody incubations were carried out overnight at 4 °C. Reversal of cross-linking was carried out at 65 °C for 3 h. The purified DNA was analysed by quantitative PCR. The primer sequences for the promoters analysed are provided in Supplementary Information, Table S4.

Immunoprecipitation and immunoblotting. Cells were washed twice with PBS and scraped into 500 μl of lysis buffer. After brief sonication, the lysate was centrifuged at 14,000*g* for 10 min at 4 °C to remove insoluble cell debris. Immunoprecipitation and immunoblotting were performed as described[22].

***In vitro* kinase assay.** Recombinant cyclin B and CDK1 (New England Biolabs) were incubated with 1 μg of purified GST–EZH2 (wild-type or mutant) in the presence of 5 μCi of [γ-^{32}P]ATP and 50 μM cold ATP in kinase buffer for 30 min at 30 °C. Alternatively, cells expressing CDK1 (and treated with *CDK1* shRNA) were labelled with 0.2 mCi ml^{-1} [^{32}P]-orthophosphate for 3 h (Fig. 2g). Reaction products were resolved by SDS–PAGE, and ^{32}P-labelled proteins were visualized by autoradiography.

***In vitro* histone methyltransferase assay.** MCF7 cells stably expressing Myc-His-tagged wild-type EZH2 and EZH2^{T487A} mutant were lysed, and tagged proteins were purified by using a combination of protein G-crosslinked Myc antibody and nickel column purification. The *in vitro* HMTase assay was performed as described[23], except for slight modifications. Briefly, 30 μl of reaction mixture containing the nickel beads attached to the purified protein, 2 μg of oligonucleosome as substrates, and 2 μCi of *S*-adenosyl-L-(methyl-^{3}H) methionine (SAM; Amersham Biosciences) as the methyl donor in methylase activity buffer (50 mM Tris-HCl at pH 8.5, 100 mM NaCl and 10 mM dithiothreitol; DTT) was incubated for 1 h at 30 °C. Proteins were resolved by 15% SDS–PAGE gel and visualized by Coomassie-blue staining and autoradiography.

ChIP-on-chip assay for EZH2. hMSCs were differentiated into osteoblasts and then cells were harvested for the ChIP-on-chip assay using NimbleGen Human ChIP 385K promoter array Two-set. The procedure was performed according to manufacturer's instructions (NimbelGen company).

Cell migration assay. Migration assays were performed in Transwell filter inserts in 24-well tissue culture plates (BD Bioscience). The polycarbonate membrane filters of Transwell contain pores 8 μm in diameter. Cells were detached at 90% confluence, washed once in PBS, and resuspended in serum-free Dulbecco's Modified Eagle Medium (DMEM). Cell suspension (250 μl) was added to inserts

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at a density of 1×10^5 cells per insert. DMEM containing 10% (v/v) fetal calf serum (FCS) was added to the lower wells. Migration was allowed to proceed at 37 °C for 16 h. Cells that did not migrate through the filters were removed using cotton swabs, and cells that migrated through the inserts were fixed and stained with crystal violet. The number of migrated cells per microscopic field (at original magnification ×200) was counted visually under a light microscope. The data were expressed as the average number of cells from five randomly selected fields. Data from three independent experiments were pooled and analysed using a two-tailed, Student's *t*-test.

Cell invasion assay. Invasion was measured using Biocoat Matrigel invasion chambers (BD Biosciences) by following the manufacturer's protocol. Briefly, cells at the 90% confluent stage were detached. DMEM containing 10% FCS was placed in the lower well, and 1×10^5 cells in 250 μl of serum-free medium were loaded to the upper chamber of the Matrigel-coated insert and incubated at 37 °C for 40 h. Cells that invaded to the lower surface of the filter were fixed and stained with crystal violet and quantified with light microscope at original magnification ×200. The data were expressed as the average number of cells from five randomly selected fields and analysed statistically using a two-tailed, Student's *t*-test. The experiments were repeated three times.

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