EZH2 engages TGFβ signaling to promote breast cancer bone metastasis via integrin β1-FAK activation

Lin Zhang1,6, Jingkun Qu1,4,6, Yutao Qi1,2, Yimin Duan1, Yu-Wen Huang1,5, Zhifen Zhou1, Ping Li1, Jun Yao1, Beibei Huang3, Shuxing Zhang2,3 & Dihua Yu1,2

Bone metastases occur in 50–70% of patients with late-stage breast cancers and effective therapies are needed. The expression of enhancer of zeste homolog 2 (EZH2) is correlated with breast cancer metastasis, but its function in bone metastasis hasn’t been well-explored. Here we report that EZH2 promotes osteolytic metastasis of breast cancer through regulating transforming growth factor beta (TGFβ) signaling. EZH2 induces cancer cell proliferation and osteoclast maturation, whereas EZH2 knockdown decreases bone metastasis incidence and outgrowth in vivo. Mechanistically, EZH2 transcriptionally increases ITGB1, which encodes for integrin β1. Integrin β1 activates focal adhesion kinase (FAK), which phosphorylates TGFβ receptor type I (TGFβRI) at tyrosine 182 to enhance its binding to TGFβ receptor type II (TGFβRII), thereby activating TGFβ signaling. Clinically applicable FAK inhibitors but not EZH2 methyltransferase inhibitors effectively inhibit breast cancer bone metastasis in vivo. Overall, we find that the EZH2-integrin β1-FAK axis cooperates with the TGFβ signaling pathway to promote bone metastasis of breast cancer.
Breast cancer is the most commonly diagnosed cancer in female individuals worldwide. About 50–70% of breast cancer patients with late-stage disease develop bone metastases that cause skeletal-related events, including pain, pathological fractures, spinal cord compression, hypercalcemia, and other complications. The treatments for bone metastasis are limited and merely palliative; standard antiresorptive agents, chemotherapy, and radiotherapy can delay or lessen skeletal-related events, but they cannot cure bone metastasis. Exploring the molecular mechanism of bone metastasis comprehensively may provide additional therapeutic strategies for patients with bone metastasis. Breast cancer bone metastasis frequently induces osteolytic lesions, which lead to massive bone resorption and bone fractures. Osteolytic bone resorption causes secretion of several growth factors, including transforming growth factor beta (TGF-β). Bone metastasis is incited by “the vicious cycle”, which designates the feed-forward cycle among cancer cells, osteoblasts, and osteoclasts in promoting both uncontrolled tumor growth and osteoclast activity.

TGF-β plays dual roles in cancer initiation and progression: it works as a tumor suppressor in premalignant cells but induces breast cancer metastasis by enhancing epithelial–mesenchymal transition, angiogenesis, and immunosuppression. Studies have well established that TGF-β is a predominant cytokine driving the feed-forward vicious cycle to promote metastatic cancer cell growth in bones. In canonical TGF-β signaling, active TGF-β binds to its receptor, TGF-β receptor type II (TGF-βRII), which binds and activates TGF-β receptor type I (TGF-βRI) on the cell membrane. TGF-βRI phosphorylates downstream signaling molecules Smad2/3, which form a complex with Smad4; the Smad2/3/4 complex is then translocated to the nucleus. The nuclear Smad2/3/4 complex works as transcription factors on the transcription of target genes.

Noncanonical TGF-β signaling works as a Smad-independent pathway through activation of p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), or phosphoinositide 3-kinase (PI3K)/AKT signaling.

EZH2 is a histone methyltransferase that serves as an enzyme subunit of the polycomb repressive complex 2. It regulates gene expression through trimethylation of histone H3 at lysine (K) 27 (H3K27me3) or as a transcription co-factor. EZH2 increases the transcription of integrin β1-encoding ITGB1 that activates a downstream effector, focal adhesion kinase (FAK). Activated FAK phosphorylates TGF-βRI and enhances the binding of TGF-βRII to TGF-βRII to activate the TGF-β signaling pathway. Our study revealed the cooperation between EZH2 and TGF-β signaling in promoting bone metastasis of breast cancer through a methyltransferase-independent mechanism, and demonstrated that targeting FAK may be an effective strategy for treatment of EZH2-induced breast cancer bone metastasis.

Results
EZH2 promotes breast cancer bone metastasis, which cannot be blocked by an EZH2 methyltransferase inhibitor. To explore the function of EZH2 in bone metastasis of breast cancer, we transfected either EZH2 shRNA or control shRNA into the MDA-MB-231 bone-seeking 231–1566 cell subline that expresses GFP and luciferase to generate the EZH2-knockdown cell lines 1566.shEZH2 and its control cell line 1566.shScr, respectively (Supplementary Fig. 1a). The sublines 1566.shEZH2 and 1566.shScr were injected, separately, into the left ventricles of nude mice. Mice injected with 1566.shEZH2 cells had significantly longer bone metastasis-free survival (P = 0.0047) and overall survival (P = 0.0024) than did mice injected with 1566.shScr cells (Fig. 1a). Bioluminescence imaging (BLI), X-ray imaging, and hematoxylin and eosin (H&E) staining of bone lesions all showed that mice injected with 1566.shEZH2 cells had fewer bone metastases than did mice injected with 1566.shScr cells on the same day post-injection (Fig. 1b).

Using the CRISPR/CAS9 system, EZH2-knockout MDA-MB-231 cell subclones (231.KO) and their control clone (231.sgCtrl) were also generated (Supplementary Fig. 1b), and one of the 231.KO subclones (231.KO#1) were stably re-expressed with wild-type EZH2 (231.KO#1.EZH2) or a pLenti control vector (231.KO#1.pLenti) (Supplementary Fig. 1c). The derived sublines 231.KO#1.EZH2 and 231.KO#1.pLenti were intracardially injected into nude mice, respectively. Mice injected with 231.KO#1.EZH2 cells were treated with a vehicle or GSK126, a potent small-molecule EZH2 methyltransferase inhibitor, whereas the control mice injected with 231.KO#1.pLenti were only treated with a vehicle. The results showed that the vehicle-treated 231.KO#1.EZH2 group had significantly poorer bone metastasis-free survival rates than did the control 231.KO#1.pLenti group (Fig. 1c, d).

Unexpectedly, the GSK126-treated 231.KO#1.EZH2 group had similar metastasis-free survival rate to that in the vehicle-treated 231.KO#1.EZH2 group (Fig. 1c, d). The data indicated that EZH2 overexpression increased the incidence of bone metastasis, which cannot be deterred by inhibiting EZH2 methyltransferase function with GSK126. Furthermore, we knocked out EZH2 in the 231–1566 cell subline, generated EZH2-knockout single clones and its control cell line (Supplementary Fig. 1d) and mixed them together as the 1566.KO cell subline. We labeled 1566.KO cells and their control cells 1566.Ctrl with GFP and luciferase, and injected them intracardially into mice to generate bone metastases. Mice injected with 1566.KO cells had significantly longer bone metastasis-free survival (P = 0.0016) and overall survival (P < 0.0001) than did mice injected with 1566.Ctrl cells (Fig. 1e, f, Supplementary Fig. 1e), and the data echo that of EZH2 shRNA knockdown in 231–1566 cells (Fig. 1a, b). EPZ-6438 (Tazemetostat) is another potent and selective EZH2 methyltransferase inhibitor, which is under several clinical trials (ClinicalTrials.gov Identifier: NCT01897571, NCT03009344) for the treatment of advanced solid tumors or lymphomas. Those 1566.Ctrl cell-injected mice were also treated with EPZ-6438 (250 mg/kg, twice/day, oral), or vehicle beginning at day 6 post-injection. Similar to findings from GSK126-treated mice bearing 231.KO#1.EZH2 bone metastasis, EPZ-6438 treatment did not deter bone metastasis incidence and progression of 1566.Ctrl cell-injected mice as there was no significant difference in the bone metastasis-free survival, overall survival and BLI images between the vehicle- versus EPZ-6438-treated groups (Fig. 1e, f, Supplementary Fig. 1e). Additionally, we pooled five of MDA-MB-231 EZH2 knockout single clones together and labeled them with GFP and luciferase (231.KO mixed) (Supplementary Fig. 1f), which were intratibially injected into nude mice with 231 cells as controls. Clearly, EZH2 knockout significantly inhibited bone metastasis outgrowth.
The above loss- and gain-of EZH2 function in vivo bone metastasis experiments demonstrated that EZH2 promoted breast cancer bone metastasis and that EZH2’s effect on bone metastasis is likely methyltransferase-independent.

To explore the mechanism of EZH2 promotion of bone metastasis, we first compared the proliferation, migration, and invasion ability of high and low EZH2-expressing MDA-MB-231 cell sublines. High EZH2-expressing cells (MDA-MB-231 and 231.sgCtrl) and low EZH2-expressing cells (231.KO #1 and #2)
had similar rates of proliferation in two-dimensional cell culture as measured using MMT assays (Supplementary Fig. 1i). However, high EZH2-expressing cells had greater migration and invasion ability in vitro than did low EZH2-expressing cells (Fig. 1g, Supplementary Fig. 1j). GSK126 treatment didn’t change cell proliferation, migration, or invasion of high EZH2-expressing MDA-MB-231 cells compared to vehicle treatment (Supplementary Fig. 1k–m). Additionally, we introduced an EZH2-methyltransferase-dead mutant EZH2-H689A into the 231.KO#1 cells to generate the stable 231.KO#1.H689A subline, along with the 231.KO#1.pLenti and 231.KO#1.EZH2 sublines (Supplementary Fig. 1c), to test EZH2 methyltransferase-independent function. Re-expression of the wild-type EZH2 (231.KO#1.EZH2) greatly increased cell migration and invasion compared to the control 231.KO#1.pLenti cells (Fig. 1b, Supplementary Fig. 1n). And re-expression of methyltransferase-dead mutant EZH2 H689A in 231.KO#1 (231.KO#1.H689A) also promoted migration or invasion just like that of re-expression of wild-type EZH2 (231.KO#1.EZH2) (Fig. 1b, Supplementary Fig. 1n). Similarly, GSK126 treatment didn’t have an inhibitory effect on migration or invasion of 231.KO#1.EZH2 cells (Fig. 1b, Supplementary Fig. 1n). These data suggested that EZH2 promoted MDA-MB-231 cell migration and invasion but did not change cell proliferation in two-dimensional cell culture. Furthermore, blocking EZH2’s histone methyltransferase function, either genetically by EZH2 H694A mutation or with EZH2 methyltransferase inhibitor GSK126, did not inhibit the migration or invasive ability induced by wild-type EZH2.

To expand the investigation of EZH2’s effect on bone metastasis, we also established CRISPR/CAS9-mediated EZH2-knockout subclones in 4T1 mouse mammary tumor cells (4T1.KO #1 and #2) (Supplementary Fig. 1o) and examined their proliferation, migration, and invasion compared with those of the control 4T1 cells. Knocking out EZH2 inhibited 4T1 cell migration and invasion but did not have an apparent effect on cell proliferation (Supplementary Fig. 1p, q). GSK126 treatment of 4T1 cells did not result in different proliferation, invasion, or migration from untreated 4T1 cells (Supplementary Fig. 1p–r). Together, our data from both MBA-MD-231 and 4T1 cells showed that EZH2 promoted cancer cell migration and invasion, but this function is unlikely dependent on EZH2’s methyltransferase activity.

EZH2 regulates the vicious cycle of breast cancer bone metastasis. The colonization and growth of cancer cells in the bone marrow are critical for bone metastasis formation. Since EZH2 knockout significantly inhibited bone metastasis outgrowth (Supplementary Fig. 1g, h), we explored the function of EZH2 in promoting metastatic breast cancer outgrowth in the bone. To mimic the vicious cycle of breast cancer bone metastasis microenvironment, we co-cultured breast cancer cells with RAW264.7 preosteoclasts and MC3T3 osteoblasts (triple co-culture) under TGFβ treatment (5 ng/mL) (Fig. 2a). The MDA-MB-231, 231.sgCtrl, 231.KO#1, and 231.KO#2 cells were pre-transfected with GFP expression vector for easy detection and quantification by flow cytometry (Supplementary Fig. 2a–c). Mature osteoclasts are detected by TRAP staining as round giant cells with three or more nuclei and they induce osteolysis to release TGFβ, which activates the vicious cycle of breast cancer bone metastasis. Six days in triple co-culture, the EZH2-knockout 231.KO#1 and 231.KO#2 cells showed significantly inhibited cell growth than MDA-MB-231 and 231.sgCtrl cells (Fig. 2b and Supplementary Fig. 2c). Also, the RAW264.7 preosteoclasts that differentiated into mature osteoclasts were significantly less in co-culture with EZH2-knockout cells (231.KO#1 and 231.KO#2) than with MDA-MB-231 or 231.sgCtrl cells (Fig. 2c). When MDA-MB-231 cells were treated with 2 μM GSK126 or a vehicle (dimethyl sulfoxide, DMSO) in triple co-culture, GSK126 did not inhibit cancer cell proliferation nor osteoclast cell maturation (Supplementary Fig. 2d, e). To further test EZH2 methyltransferase function in vicious cycle of breast cancer bone metastasis, 231.KO#1.pLenti, 231.KO#1.EZH2, and 231.KO#1.H689A cells were compared in triple co-culture. Similar to the wild-type EZH2 re-expressing 231.KO#1.EZH2 cells, 231.KO#1.H689A cells that re-expressing EZH2 methyltransferase-dead mutant H689A had enhanced tumor cell proliferation and osteoclast maturation compared to 231.KO#1.pLenti cells (Fig. 2d, e). In addition, EZH2 re-expressing 231.KO#1.EZH2 cells with or without GSK126 treatment showed similarly increased tumor cell proliferation and osteoclast maturation compared to 231.KO#1.pLenti cells (Fig. 2d, e). Likewise, we performed triple co-culture experiments with 4T1 cells and EZH2-knockout 4T1 cell sublines (4T1.KO #1 and #2) as well as treating 4T1 cells with GSK126, and had consistent findings as those from the MDA-MB-231 cell sublines. Mainly, (i) high EZH2-expressing 4T1 cells possessed a growth advantage and induced osteoclasts maturation more than EZH2-knockout cells did; (ii) GSK126 didn’t inhibit 4T1 cell proliferation or RAW264.7 preosteoclasts maturation in the triple co-culture (Supplementary Fig. 2f, g).

Next, we examined whether EZH2 promoted bone metastasis outgrowth in vivo by increasing tumor cell proliferation or and inhibiting apoptosis by IHC staining of Ki67 and cleaved caspase 3, which showed that the bone metastasis of EZH2 knockdown 1566.shEZH2 cells had significantly decreased proliferation and increased apoptosis compared to that of 1566.shScr cells (Supplementary Fig. 2h). Furthermore, we intratibially injected 231.KO#1.EZH2 and 231.KO#1.H689A cells into mice and monitored the bone metastasis outgrowth. We found that 231.KO#1.EZH2 and 231.KO#1.H689A cells induced bone metastasis lesions similarly (Fig. 2f). Together, data from both cell models and in vivo experiments indicated that EZH2 promoted the vicious cycle of breast cancer bone metastasis, which cannot be blocked by EZH2 methyltransferase inhibitor or the EZH2 H689A methyltransferase dead mutation.

Parathyroid hormone-like hormone (PTHHL, also named PTHRP) is an essential mediator of breast cancer bone metastasis, and metastatic cancer cells secrete PTHHL into the bone microenvironment to activate osteolysis. Knockout of EZH2 in both MDA-MB-231 and 4T1 cells reduced their PTHHL mRNA expression under TGFβ treatment as measured by qRT-PCR (Fig. 2g, h), whereas GSK126 treatment of 4T1 cells didn’t reduce Pthlh mRNA expression (Fig. 2h). Besides PTHHL, IL-8 is a cytokine that also regulates osteolysis in breast cancer bone metastasis. Knockout of EZH2 in MDA-MB-231 and 4T1 cells also inhibited their IL-8 mRNA expression, but GSK126 treatment didn’t change it (Supplementary Fig. 2i, j). These data indicated that EZH2 facilitates PTHHL and IL-8 expressions, which can mediate the vicious cycle of breast cancer bone metastasis.

EZH2 increases pS465/467-Smad2 and pY397-FAK levels in response to TGFβ stimulation. PTHHL is a well-known TGFβ downstream gene regulated by the p-Smad2/3 transcription factor complex or p38 MAPK. To further explore how EZH2 facilitates PTHHL and TGFβ signaling in breast cancer cells, we detected pS465/467-Smad2 and pT180/Y182-p38 MAPK levels in MDA-MB-231 sublines. In response to TGFβ stimulation, knockout of EZH2 in MDA-MB-231 cells inhibited pS465/467-Smad2 levels without significant changes of total Smad2, Smad3,
Fig. 2 EZH2 regulates the vicious cycle of breast cancer bone metastasis. a Model of triple co-culture of breast cancer cells with preosteoclasts and osteoblasts. Malignant RAW 264.7 preosteoclasts were seeded into the wells of six-well plates. GFP-labeled breast cancer cells and MC3T3 osteoblasts were seeded into Millicell Hanging Cell Culture Inserts (Millipore) in the six-well plates and treated with TGFβ (5 ng/mL). b Quantification of MDA-MB-231, 231.sgCtrl, 231.KO#1, and 231.KO#2 cells after co-culture with osteoclasts and MC3T3 osteoblasts treated with TGFβ (5 ng/mL) for 6 days. Data are presented as means ± S.E.M. t-test (two-sided). Three biologically independent experiments. c Representative staining images and quantification of mature TRAP+ osteoclasts after culture with MC3T3 osteoblasts and the indicated cancer cells, and treatment of them with TGFβ (5 ng/mL) for 6 days. The arrows indicate multinuclear mature TRAP+ osteoclasts. Scale bars, 200 μm. Data are presented as means ± S.E.M. t-test (two-sided). Ten random vision fields examined over three biologically independent experiments. d Quantification of 231.KO#1.pLenti cells treated with vehicle, 231.KO#1.EZH2 cells treated with vehicle, 231.KO#1.EZH2 cells treated with 2 μM GSK126, 231.KO#1.H689A cells treated with vehicle, after co-culture with osteoclasts and MC3T3 osteoblasts treated with TGFβ (5 ng/mL) for 6 days. Data are presented as means ± S.E.M. t-test (two-sided). Three biologically independent experiments. e Representative staining images and quantification of mature TRAP+ osteoclasts after co-culture with MC3T3 osteoblasts and the indicated cancer cells and treatment of them with TGFβ (5 ng/mL) for 6 days. The arrows indicate multinuclear mature TRAP+ osteoclasts. Scale bars, 200 μm. Data are presented as means ± S.E.M. t-test (two-sided). Three biologically independent experiments. f Representative bioluminescence image (BLI), X-ray images and quantification of BLI signals of bone-metastatic lesions in the two subgroups of mice (n = 5 in each group) intratibially injected with 231.KO#1.EZH2 or 231.KO#1.H689A cells 5 weeks post-injection. Data are presented as means ± S.E.M. t-test (two-sided). The arrows indicate osteolytic bone lesions in X-ray images. g qRT-PCR analysis of PTHLH mRNA expression in the indicated cells treated with a vehicle or TGFβ (5 ng/mL, 2 h). Data are presented as means ± S.E.M. t-test (two-sided). N.S. not significant. Three biologically independent experiments. h qRT-PCR analysis of Pthh mRNA expression in the indicated cells treated with a vehicle or TGFβ (5 ng/mL, 2 h). Data are presented as means ± S.E.M. t-test (two-sided). Three biologically independent experiments. All P values are indicated in the figures. The TGFβ used in all experiments in this study is TGFβ1.
or Smad4 protein expressions (Fig. 3a and Supplementary Fig. 3a). Knockout of EZH2 didn't change the level of pT180/Y182-p38 MAPK, suggesting that EZH2 does not regulate TGFβ-p38 MAPK signaling (Fig. 3a). Knockdown of EZH2 by shRNAs (shEZH2#3 and shEZH#4) yielded similar results in MDA-MB-231 sublines (Supplementary Fig. 3b). To examine whether EZH2-methyltransferase function is involved in regulation of pS465/467-Smad2 levels, we measured pS465/467-SMAD2 levels in 231.KO#1 sublines that have re-expression of the control vector, wild-type EZH2, or H689A EZH2, in response to TGFβ (Fig. 3b). Like wild-type EZH2 re-expressing cells, H689A EZH2 re-expression also increased the level of pS465/467-Smad2 compared with the control vector (Fig. 3b). Furthermore, GSK126 treatment had no significant impact on increased pS465/467-Smad2 by TGFβ treatment (Supplementary Fig. 3c).

To gain insight into how EZH2 activates Smad2 signaling, we first measured the expressions of TGFβRI and TGFβRII, the TGFβ receptors upstream of pS465/467-Smad2, using flow cytometry or western blotting, and detected no significant changes in EZH2-knockout sublines (Supplementary Fig. 3d–f).

Next, we performed reverse-phase protein array (RPPA) to profile protein expression changes in MDA-MB-231 and 231.sgCtrl cells versus those in EZH2-knockout MDA-MB-231 cells (231.KO #1 and #2) with or without TGFβ treatment. RPPA revealed that 228 proteins were downregulated and 194 proteins were upregulated in the two EZH2-knockout cell lines compared to that in MDA-MB-231 and 231.sgCtrl cells (Supplementary Data 1), and gene ontology molecular functional analysis showed that the dramatically downregulated and upregulated proteins were kinases, including tyrosine kinases (Fig. 3c, and Supplementary Fig. 3g, h).
Notably, phosphorylation of tyrosine 397 on FAK (pY397-FAK) was significantly reduced in the EZH2-knockout cells (Fig. 3c). FAK is a non-receptor tyrosine kinase that regulates the survival, proliferation, migration, and invasion of cancer cells and can impact on cancer development and progression24,25. Unlike the FAK upstream kinase Src, whose function in bone metastasis is well-documented26–28, the function of FAK in bone metastasis is unclear. We thus validated RPPA data by western blotting, which showed that knocking out EZH2 in MDA-MB-231 and MCF7 cells inhibited both pY397-FAK and pS465/467-Smad2 levels under TGFβ treatment (Fig. 3d and Supplementary Fig. 3i). GSK126 treatment of MDA-MB-231 and 231–1566 cells with or without TGFβ stimulation didn’t change pY397-FAK or pS465/467-Smad2 levels compare to vehicle-treated cells (Supplementary Fig. 3j). Collectively, these data suggested that EZH2 functions to activate FAK and Smad2 signaling under TGFβ stimulation.

To examine whether increased pY397-FAK is related to enhanced pS465/467-Smad2, we treated MDA-MB-231 cells with FAK inhibitors (FAKi, VS-4718, or VS-6063) at different concentrations (1–10 μM), followed with TGFβ treatment (5 ng/ml, 2 h), then detected pS465/467-Smad2. FAKi treatment diminished pS465/467-Smad2 level and reduced PTHLH mRNA expression even under TGFβ stimulation (Fig. 3e and Supplementary Fig. 3k, l). Additionally, knocked down FAK using shRNAs (shFAK#2 or shFAK#3) in MDA-MB-231 cells also inhibited pS465/467-Smad2 levels (Fig. 3f). In the bone-seeking 231–1566 subline, knocking down FAK alone with siRNAs (siFAK#1 or siFAK#2) didn’t change pS465/467-Smad2 levels (Supplementary Fig. 3m); however, doubly knocking down FAK and its closely related kinase PYK2 (or FAK2) with siRNAs (siPYK2#4, siPYK2#9, or siPYK2#50) dramatically reduced pS465/467-Smad2 levels (Supplementary Fig. 3n). Doubly knocking down FAK and PYK2 also inhibited PTHLH mRNA expression (Supplementary Fig. 3n). These data indicated that activation of FAK family kinases by EZH2 increased the phosphorylation of S465/467-Smad2 and activated the TGFβ/Smad2/PTHLH pathway in breast cancer cells.

pY397-FAK induces TGFβRI tyrosine phosphorylation that enhances its binding to TGFβRII in response to TGFβ. We further explored the mechanism of how EZH2-mediated FAK activation induces pS465/467-Smad2. Smad7 can block the TGFβRI-induced pS465/467-Smad229, but knocking down EZH2 did not change Smad7 expression (Fig. 3d). Thus, we assessed whether FAK regulates TGFβRI and TGFβRII expressions in MDA-MB-231 cells. Knocking down FAK didn’t change the protein expressions of TGFβRI or TGFβRII (Supplementary Fig. 4a, b). Next, we tested whether FAK can bind to Smad2 to phosphorylate Smad2 by immunoprecipitation (IP) of FAK followed with western blotting of Smad2, which didn’t show detectable binding (Supplementary Fig. 4c). Surprisingly, FAK IP brought down TGFβRII, not TGFβRI, with or without TGFβ exposure (Fig. 4a). Additionally, blocking FAK kinase activity by FAKi VS-4718 treatment reduced the binding of FAK to TGFβRII (Fig. 4b). Since TGFβ treatment induces TGFβRII binding to TGFβRII (Supplementary Fig. 4d) and, consequently, increased pS465/467-Smad2 levels, we postulated that pY397-FAK may phosphorylate TGFβRII that increases the binding affinity of TGFβRII under TGFβ stimulation, leading to activation of Smad2 signaling. To test this, MDA-MB-231 cells were treated by the FAKi VS-4718 or had FAK knockdown by shRNAs (shFAK#21 or shFAK#3), and treated with TGFβ. After collecting cell lysates, we performed TGFβRI IP followed by western blotting of TGFβRII, which showed that FAK inhibition dramatically reduced the binding of TGFβRI to TGFβRII in response to TGFβ stimulation (Fig. 4c and Supplementary Fig. 4e, f). To explore whether FAK tyrosine kinase can phosphorylate TGFβRII, we performed IP to pull down TGFβRII from MDA-MB-231 cells (231.TGFβRII) or HEK 293FT cells transfected with exogenous FLAG-tagged wild-type TGFβRII (293FT.TGFβRII), and then western blotting with anti-phospho-tyrosine antibodies. We detected tyrosine phosphorylation on TGFβRII (Supplementary Fig. 4g, h), which were reduced by FAKi VS-6063 treatment (Fig. 4d). The data indicated that activated FAK can induce tyrosine phosphorylation of TGFβRII. Next, we performed mass spectrometric analysis to locate the site of FAK-induced tyrosine phosphorylation on TGFβRII. We identified an unreported TGFβRII phosphorylation site at tyrosine 182 (pY182), which is located in the glycine and serine residues enriched-domain (GS domain)30 (Fig. 4e). Notably, it is known that after TGFβ binding, activated TGFβRII phosphorylates TGFβRII in the GS domain to activate TGFβRII kinase function and the transduction of TGFβ signals31.

Structural analysis revealed that Y182 of TGFβRII is highly exposed for potential phosphorylation (Fig. 4f), and it is close to two threonine and one serine sites (T185, T186, S187) in TGFβRII (Fig. 4g). Upon phosphorylation, the distance of the negatively charged phosphate group of TGFβRII to positively charged K381 of TGFβRII becomes much closer (2.1 Å), therefore, significantly enhancing binding of TGFβRII to TGFβRII through increased charge–charge interactions with positively charged K381, as well as Mg2+ coordinated with ATP (Supplementary Fig. 4i right and Fig. 4g right). Consequently, such increased TGFβRII binding to TGFβRII may further promote the phosphate transfer from the TGFβRII-bounded ATP to T185, T186, and S187 in the GS domain of TGFβRII (Fig. 4g). To determine whether phosphorylation of Y182 at TGFβRII changes the binding affinity of TGFβRII to TGFβRII, we stably expressed FLAG-tagged wild-type TGFβRII (RI-WT-FLAG), a non-phosphorylatable Y182F mutant TGFβRII (RI-YF-FLAG), or a phosphomimetic Y182D mutant TGFβRII (RI-YD-FLAG) in HEK 293FT cells. The wild-type TGFβRII, mutant TGFβRII Y182F, or TGFβRII Y182D in these cells were pulled down with an anti-FLAG antibody after TGFβ treatment followed by western blotting of TGFβRII. We found that the non-phosphorylatable TGFβRII-Y182F mutant had reduced binding to TGFβRII, compared with wild-type TGFβRII and TGFβRII-Y182D mutant (Supplementary Fig. 4j, k); phosphomimetic TGFβRII-Y182D mutant had slightly increased binding to TGFβRII, compared with wild-type TGFβRII (Supplementary Fig. 4l). Evidently, the phosphorylation of TGFβRII at Y182 enhanced TGFβRII binding to TGFβRII in response to TGFβ stimulation, which is consistent with our protein docking analysis.

EZH2 increases the FAK upstream ITGB1 expression. FAK signaling is initiated by integrin-mediated cell adhesions. Integrins (such as β1 or β3) can facilitate FAK autophosphorylation at tyrosine 397, which increases the catalytic activity of FAK24,33. To understand the underlying mechanism of EZH2-induced pY397-FAK, we investigated whether and how EZH2 regulates expression of integrins β1 or β3. Since EZH2 methyltransferase activity is not required for increasing pY397-FAK (Supplementary Fig. 3j) and we recently reported that EZH2 can function as a transcription co-factor of RNA Pol II to upregulate mRNA transcription34, we examined whether EZH2 also regulate RNA Pol II transcription of genes encoding β1, β3, or other genes that may regulate pY397-FAK. We analyzed our chromatin IP
sequencing (ChIP-seq) dataset (GSE188640) to compare RNA Pol II occupancy of gene promoters between EZH2-expressing MDA-MB-231 cells and 231.KO#1 cells in which EZH2 was knocked out. The result showed that knocking out EZH2 led to reduced binding of RNA Pol II to promoter regions of at least 470 genes (Supplementary Data 2). Among these, binding of RNA Pol II to ITGB1 (encoding integrin β1) promoter was substantially decreased, whereas binding to ITGB3 (encoding integrin β3) showed little changes (Fig. 5a and Supplementary Fig. 5a). Consistently, qRT-PCR showed that knockdown and knockout of EZH2 downregulated ITGB1 mRNA expression (Fig. 5b, c), resulting in decreased integrin β1 protein expression (Supplementary Fig. 5b, c), while knockdown of EZH2 had no significant effect on β3 mRNA expression (Supplementary Fig. 5d). Also, ITGB1 promoter-driven luciferase reporter gene assays showed that ITGB1 promoter activity was higher in EZH2-expressing MDA-MB-231 and 231.sgCtrl cells than that in EZH2-null cells (231.KO#1 and 231.KO#2) (Fig. 5d).

To detect the EZH2 and RNA Pol II bindings to the ITGB1 promoter in MDA-MB-231 versus 231.KO#1 cells, we performed ChIP-seq analysis with RNA Pol II (Supplementary Fig. 5b, c) and EZH2 (Supplementary Fig. 5d) in MDA-MB-231 versus 231.KO#1 cells. We found that EZH2 negatively regulated the ITGB1 promoter activity by genome-wide chromatin immunoprecipitation with DNA microarray analysis (ChIP-seq). The result showed that EZH2 binding to ITGB1 promoter led to reduced RNA Pol II binding, resulting in decreased ITGB1 mRNA expression (Fig. 5b, c).
ChIP-qPCR using a series of PCR primers that bind to various regions of the ITGB1 promoter from −2.6 kb upstream of (primer P1) to near (primer P5), the ITGB1 transcription start site (Fig. 5e, top). In MDA-MB-231 cells, EZH2 was recruited to the ITGB1 promoter from P1 to P5 loci, and expectedly, in 231.KO#1 cells, binding of EZH2 to these loci of ITGB1 promoter was lost (Fig. 5e, bottom). Our ChIP-EZH2-qPCR assays showed that EZH2 binds to ITGB1 promoter almost at the level of EZH2 binding to HOXA9B, a well-known methyltransferase substrate of EZH2, with no binding to a non-substrate gene promoter.
we questioned whether integrin activated FAK bound to and phosphorylated TGFβ1 in the same promoter regions of ITGB1 promoter were similar in control 231.shScr cells versus EZH2-KO, whereas the cytoplasmic domain of integrin β1 may bind with TGFβ1 through TGFβ1, which further demonstrated the cross interactions between the TGFβ1/TGFβRI pathway and the integrin β1/FAK pathway. Moreover, our molecular docking between TGFβRI/TGFβ1 and integrin αvβ1 complex using the ClusPro web server showed that TGFβRI interacts with integrin αvβ1 among their ectodomains and this interaction requires TGFβ1 (Supplementary Fig. 5k). Interestingly, our IP of TGFβRI from untreated MDA-MB-231 cells followed with western blotting of integrin β1 showed that TGFβRI still can bind with integrin β1 with TGFβ1 (Supplementary Fig. 5k), conceivably via cytoplasmic domain. The binding between TGFβRI and integrin β1 without TGFβ1 stimulation was reduced by FAK inhibitor treatment (Supplementary Fig. 5l), implying that activated FAK can mediate the cytoplasmic binding of TGFβRI with integrin β1. These data suggested that the ectodomain of integrin β1 may bind with TGFβRI through TGFβ1, whereas the cytoplasmic domain of integrin β1 may bind with TGFβRI through activated FAK (Supplementary Fig. 5m).

To further examine whether integrin β1 regulates Y182 phosphorylation of TGFβ1, we knocked down ITGB1 by siRNA in HEK 293FT cells expressing FLAG-tagged wild-type TGFβRI. After pulling down FLAG-TGFβRI, western blotting of tyrosine phosphorylation of TGFβRI detected a dramatic reduction by ITGB1 knockdown (Supplementary Fig. 5n), indicating that integrin β1, as an EZH2 downstream effector, regulates Y182 phosphorylation of TGFβRI. Furthermore, we investigated whether integrin β1 has a similar effect on the pS465/467-Smad2 level as EZH2 and pY397-FAK. We knocked down integrin β1 by (Supplementary Fig. 5e). Markedly, RNA Pol II bound well to P1 to P5 loci within the ITGB1 promoter that overlapped with EZH2 binding loci in MDA-MB-231 cells, and RNA Pol II binding to the ITGB1 promoter was also lost in EZH2-knockout 231.KO#1 cells (Fig. 5f), indicating EZH2 is required for RNA Pol II binding to the ITGB1 promoter. Similarly, shRNA-mediated knocking down of EZH2 in 231.shEZH2#3 and 231.shEZH2#4 cells also reduced the RNA Pol II binding at P1 to P4 loci of the ITGB1 promoter compared to control 231.shScr cells (Fig. 5g), which paralleled with the reduced EZH2 binding (Supplementary Fig. 5f). Expectedly, EZH2 knockdown in 231.shEZH2#3 and 231.shEZH2#4 cells reduced both EZH2 binding and H3K27me3 binding to, but increased RNA Pol II binding at, the HOXA9B promoter, compared to control 231.shScr cells; However, H3K27me3 binding to the ITGB1 promoter were similar in control 231.shScr cells versus EZH2-knockdown cells (Supplementary Fig. 5g), further indicating EZH2 regulated ITGB1 independent of its methyltransferase function. Taken together, both EZH2 and RNA Pol II bind to the same promoter regions of ITGB1, and EZH2 is likely functioning as a co-factor of RNA Pol II to upregulate ITGB1 transcription independent of its methyltransferase function.

Since integrin β1 is responsible for FAK activation and activated FAK bound to and phosphorylated TGFβRI (Fig. 4a, d, e), we questioned whether integrin β1 can bind to TGFβRI in the same complex. IP integrin β1 followed by western blotting of TGFβRI and reverse IP TGFβRI followed by western blotting of integrin β1 explicitly showed that integrin β1 can bind to TGFβRI in MDA-MB-231 cells (Supplementary Fig. 5h, i), which further demonstrated the cross interactions between the TGFβ1/TGFβRI pathway and the integrin β1/FAK pathway. Moreover, our molecular docking between TGFβRI/TGFβ1 and integrin αvβ1 complex using the ClusPro web server showed that TGFβRI interacts with integrin αvβ1 among their ectodomains and this interaction requires TGFβ1 (Supplementary Fig. 5k). Interestingly, our IP of TGFβRI from untreated MDA-MB-231 cells followed with western blotting of integrin β1 showed that TGFβRI still can bind with integrin β1 without TGFβ1 (Supplementary Fig. 5k), conceivably via cytoplasmic domain. The binding between TGFβRI and integrin β1 without TGFβ1 stimulation was reduced by FAK inhibitor treatment (Supplementary Fig. 5l), implying that activated FAK can mediate the cytoplasmic binding of TGFβRI with integrin β1. These data suggested that the ectodomain of integrin β1 may bind with TGFβRI through TGFβ1, whereas the cytoplasmic domain of integrin β1 may bind with TGFβRI through activated FAK (Supplementary Fig. 5m).

To further examine whether integrin β1 regulates Y182 phosphorylation of TGFβ1, we knocked down ITGB1 by siRNA in HEK 293FT cells expressing FLAG-tagged wild-type TGFβRI. After pulling down FLAG-TGFβRI, western blotting of tyrosine phosphorylation of TGFβRI detected a dramatic reduction by ITGB1 knockdown (Supplementary Fig. 5n), indicating that integrin β1, as an EZH2 downstream effector, regulates Y182 phosphorylation of TGFβRI. Furthermore, we investigated whether integrin β1 has a similar effect on the pS465/467-Smad2 level as EZH2 and pY397-FAK. We knocked down integrin β1 by
two siRNAs or blocked integrin β1 signaling with antibodies in MDA-MB-231 cells, treated cells with vehicle or TGFβ, and detected significantly inhibited pS465/467-Smad2 levels by targeting integrin β1 (Supplementary Fig. 5o, p). Additionally, re-overexpressing ITGB1 in 231 EZH2 knockdown subline or knockdown subline rescued the pS465/467-Smad2 level (Supplementary Fig. 5q, r). Both loss-of- and gain-of-ITGB1 function experiments indicated that integrin β1 can mediate EZH2’s regulatory function on TGFβ signaling. Moreover, IHC staining of integrin β1 and pY397-FAK in the bone metastases lesions of control 1566.shScr versus EZH2 knockdown 1566.shEZH2 cells (Fig. 1a, b) showed that EZH2 knockdown led to lower ITGB1 expression and pY397-FAK level in vivo (Supplementary Fig. 5s, t), which further demonstrated that integrin β1 and pY397-FAK are downstream effectors of EZH2.

Treatment with a clinically applicable FAK inhibitor blocks EZH2-induced breast cancer bone metastasis. Our above findings indicated that EZH2, via upregulating integrin β1 transcription, activated FAK, which activated the TGFβ/Smad2 pathway to increase bone metastasis. Our findings prompted us to test FAK inhibitor for treatment of bone metastases of high EZH2 expressing breast cancers. For bone metastasis outgrowth model, GFP- and luciferase-labeled MDA-MB-231 cells, which have relatively high EZH2 expression among tested breast cancer cell lines (Supplementary Fig. 6a), were intratibially injected into nude mice. We treated these mice with FAKi VS-6063 (50 mg/kg, twice a day, oral gavage), which is currently tested in clinical trials for treating patients with advanced lymphoma or solid tumors (ClinicalTrials.gov Identifier: NCT04439331, NCT03875820). To validate that EZH2-induced breast cancer bone metastasis outgrowth is independent of its methyltransferase function, a group of mice was treated with EZH2 methyltransferase inhibitor GSK126 (100 mg/kg, once a day, i.p. injection). The resulting bone metastasis outgrowth were detected using BLI, which confirmed that GSK126 treatment did not block tumor outgrowth in the bones (Fig. 6a). Excitingly, treatment with the FAKi VS-6063 significantly impeded the outgrowth of bone tumors compared to the control group (P = 0.0442) (Fig. 6a) and did not induce significant side effects (Supplementary Fig. 6b–e). IHC staining of bone metastasis showed that FAKi VS-6063, but not GSK126, significantly reduced pS465/467-Smad2 (P = 0.0066) level and PTHLH expression (P = 0.0074) in the bone metastases and both drugs effectively inhibited their targets (Fig. 6b, c and Supplementary Fig. 6f).

Finally, we examined the GSE2603 dataset and validated that EZH2 expression was negatively correlated with bone metastasis-free survival in breast cancer patients (r = −0.2394, P = 0.03) (Fig. 6d), suggesting that high EZH2 expression in primary breast tumors produces a high risk of developing bone metastasis in patients. We also examined the correlation between the expression of EZH2 and the downstream effector PTHLH in bone metastasis tissues obtained from breast cancer patients in the GSE14020 dataset. We found that EZH2 mRNA expression was positively correlated with PTHLH mRNA expression in patients’ bone metastases (r = 0.4630, P = 0.005) (Fig. 6e) but not in metastases to other organ sites (e.g., lung, liver, brain metastases; r = 0.2452, P = 0.097) (Supplementary Fig. 6g). This unique effect of EZH2 in promoting bone metastasis may result from dramatically higher TGFβ expression in bone metastasis than that in metastases of other organs and in primary mammary tumors (Supplementary Fig. 6h), i.e., the enriched TGFβ effectively activate the EZH2/integrin β1/FAK/p-Smad2 axis to upregulate PTHLH in bone metastasis. Most importantly, the clinical data confirmed that EZH2 high expression can increase PTHLH expression that promotes bone metastasis in patients.

Discussion
As described herein, we revealed a mechanism of how EZH2 promotes breast cancer bone metastasis. Specifically, EZH2 works as a transcription co-factor of RNA Pol II to increase ITGβ1 gene transcription; the increased integrin β1 induces phosphorylation of Y397 on FAK leading to FAK activation; activated pY397-FAK phosphorylates TGFβRI at Y182 that increases TGFβRI’s binding affinity for TGFβRII in response to TGFβ exposure, thereby triggering pS465/467-Smad2 that induces the downstream effector PTHLH; PTHLH accelerates osteolysis leading to more TGFβ release, and thus driving the feed-forward vicious cycle of breast cancer bone metastasis outgrowth (Fig. 7). Since FAK and TGFβ enhances epithelial–mesenchymal transition and cell migration,8,38, EZH2-induced FAK/TGFβ signaling activation is also an underlying mechanism of the strong migration and invasion ability of EZH2 high expressing breast cancer cells. Activation of TGFβ signaling by FAK-induced phosphorylation has a critical and distinct effect in enhancing bone metastasis, partially due to the TGFβ-enriched bone microenvironment.

Although the function of TGFβ signaling in the bone metastasis of breast cancer is well-known, the cross-talk between the integrin/FAK and TGFβ pathways is not well-documented. Integrin β6 and β8 were reported to bind with TGFβ latency-associated peptide (LAP) and convert the latent TGFβ to the active form of TGFβ39,40. And it was reported that TGFβ activated FAK through integrin β3 or β1 and leading to p38 MAPK activation in renal cell carcinoma and hepatocarcinoma cells.41,42 In the present study, we found that integrin β1-FAK is involved in the classical TGFβ/Smad-dependent pathway rather than the p38 MAPK pathway. Also, our data on the binding between integrin β1 and TGFβRII suggested that TGFβ may activate FAK through the TGFβRI- integrin β1 complex as targeting integrin β1 inhibited FAK activating under TGFβ treatment. Administering FAKi and genetically rendering FAK deficient in breast cancer cells abrogated the interaction between TGFβRI and TGFβRII and thereby blocked phosphorylation of Smad2 and expression of its downstream effector PTHLH. Our study demonstrated that the integrin/FAK and TGFβ/TGFβRI/TGFβRII pathways can cross talk and critically cooperate in driving the feed-forward vicious cycle of breast cancer bone metastasis.

It was reported that FAK and integrin β3 bind to TGFβRII in stellate hepatic cells43 and breast cancer cells44. Here, we uncovered that in EZH2 high expressing breast cancer cells, FAK and integrin β1 bound to TGFβRI rather than TGFβRII and that FAK phosphorylated TGFβRII. Little is known about tyrosine phosphorylation sites in TGFβRI and their functions, although several serine phosphorylation sites have been reported in TGFβRI45. Our mass spectrometry analysis identified a previously unreported tyrosine phosphorylation site at Y182 in the GS domain of TGFβRI. Our protein structure analysis and IP/western blotting experiments showed that the Y182 of TGFβRI is important for regulating the binding of TGFβRI to TGFβRII and subsequent TGFβ/Smad2 pathway activation. Interestingly, Y182 is conserved in the GS domains of several activin type I receptors of the TGFβ superfamily, such as ALK4 and ALK7, but not in the GS domains of bone morphogenetic protein (BMP) type I receptors of the TGFβ superfamily, such as ALK2. However, it was reported that R206H mutation at the GS domain of ALK2, led to constitutive activation of this receptor and BMP signaling46, which can crosstalk to TGFβ signaling in bone formation47. This report and our findings indicated that...
EZH2 is a classic epigenetic protein that silences tumor suppressors through H3K27me3\(^3\). Recently, its noncanonical functions in the development of various cancers are gaining increasing attentions. For example, EZH2 can methylate non-histone substrates, such as Jarid2, STAT3, RORa, and PLZF, to regulate their transcription function or protein stability\(^48\)\(^\text{-}\)\(^51\). EZH2 also has functions independent of its histone methyltransferase activities. For example, EZH2 forms a complex with RelA and RelB to activate nuclear factor \(\kappa B\) signaling in estrogen receptor-negative breast cancer cells\(^5\)\(^2\)\(^\text{-}\)\(^5\)\(^4\) and activates androgen receptor gene transcription through binding at the androgen receptor promoter in prostate cancer cells\(^5\)\(^3\)\(^,\)\(^5\)\(^4\). We recently reported that EZH2 can function as a methyltransferase-independent transcription factor to upregulate \(c\)-\(JUN\) expression that induced G-CSF to facilitate the brain infiltration of immunosuppressive neutrophils\(^3\)\(^4\). In the present study, we found that EZH2 upregulated \(ITGB1\) transcription in breast cancer cells by functioning as a transcriptional co-factor of RNA Pol II to facilitate its binding to the \(ITGB1\) promoter. Thus, \(ITGB1\) is a substrate regulated by EZH2 methyltransferase-independent activities.

EZH2 is highly expressed in various human malignancies and regulates tumor progression. Therefore, it is regarded as an attractive therapeutic target in cancer patients\(^3\)\(^5\). However, targeting EZH2 with methyltransferase inhibitors has not always proven to be beneficial in clinical trials\(^3\)\(^5\)\(^\text{-}\)\(^5\)\(^8\), partially because of the EZH2 methyltransferase-independent functions in promoting cancer development as mentioned above. In the present study, we found that small-molecule EZH2 inhibitors cannot block MDA-MB-231 cell-induced bone metastasis. However, targeting EZH2 downstream effector FAK with clinically applicable kinase inhibitors have striking effects on blocking breast cancer bone metastasis.

EZH2 inhibitors were reported to inhibit breast cancer lung metastasis in mouse models\(^5\)\(^9\)\(^,\)\(^6\)\(^0\), whereas EZH2 inhibitor EPZ-6438 failed to block but promoted bone metastasis in experimental models\(^6\)\(^1\). These contradictory effects of EZH2 on lung metastasis versus bone metastasis suggest that EZH2 inhibitors’ effects are dependent on the tumor microenvironment. Bone metastasis is reported to have significantly lower EZH2 activity compared to the lung metastases\(^6\)\(^1\), suggesting that targeting EZH2 methyltransferase activity by EZH2 inhibitors in the unique bone microenvironment might not yield inhibitory efficacies on bone metastasis. In addition, the methyltransferase-independent EZH2-Integrin \(\beta\)1-FAK-TGF\(\beta\) pathway identified in this study might be more predominant in the TGF\(\beta\)-enriched bone metastasis microenvironment, but not in the lung metastasis microenvironment of lower TGF\(\beta\) levels. Therefore, the efficacy of targeting EZH2 methyltransferase varies in different metastasis organs.

Since EZH2 plays distinct functions in different types of cancer and in metastases of different organs, targeting downstream effectors of EZH2, or EZH2’s enzyme function should be carefully evaluated. We found that FAK is a downstream effector of EZH2 in the vicious cycle of breast cancer bone metastasis. Thus, treatment with a FAKi combined with standard antiresorptive agents, chemotherapy, or radiotherapy may provide added benefit to breast cancer patients who suffer from bone metastasis.

**Methods**

**Reagents and plasmids.** Antibodies against EZH2 (#5236), H3 (#4499), pS465/467-Smad2, Smad2 (#5339), Smad3 (#9513), Smad4 (#46535), pT180/Y182-p38 (#4511), p38 (#8690), pY397-FAK (#3283), FAK (#3285), FLAG (#14793) and cleaved caspase 3 (#9664) were purchased from Cell Signaling Technology. Antibodies against \(\beta\)-actin (A5441) was purchased from Sigma-Aldrich. Antibodies against TGF\(\beta\)RI (ab31013 for IP and western blotting), TGF\(\beta\)RII (ab189498), and Ki67 (ab15580) were purchased from Abcam. The antibody against TGF\(\beta\)RII (#ABF17-I for western blotting) and H3K27me3 (Millipore #07-449) were purchased from Millipore. Antibodies against integrin \(\beta\)1 (sc-8978, sc-9970), and IgG (sc-2025, sc-2027) were purchased from Santa Cruz Biotechnology. Antibodies against RNA Pol II (NB200-598) were purchased from Novus Biologicals. The antibody against phospho-tyrosine (#610000) was purchased from BD Biosciences. Antibodies against PTHLH (#MAB6734) and Smad7 (#MAB2029) were purchased from R&D Systems. The antibody against TGF\(\beta\)1
 western blotting and immunoprecipitation (IP) were performed as described previously. Briefly, for western blotting, cells were lysed in lysis buffer (5 M urea, 10% sodium dodecyl sulfate (SDS), DNase-free water: 1:1) and then sonicated. The lysates were collected for western blotting analysis. Proteins were separated using SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. After each membrane was blocked with 5% skim milk for 1 h, it was incubated with various primary antibodies overnight at 4°C followed by incubation with secondary antibodies for 1 h at room temperature before being visualized with enhanced chemiluminescence reagent. For IP, cells were washed twice with phosphate-buffered saline and scraped into IP lysis buffer (1% Triton X-100, 130 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EGTa, 1 mM EDTA, 0.5 mM sodium orthovanadate, 0.4 mM phenylmethylsulfonyl fluoride, 0.5% NP-40). The total cell lysates were preclarified via incubation with protein G-agarose beads (Sigma; #1124323000) for 2 h at 4°C. After preclarification, lysates were incubated with the primary antibody overnight at 4°C and then with protein G-agarose beads for 4 h. The beads were washed three times with IP buffer three times, and the immunocomplex was extracted from agarose and detected using SDS-polyacrylamide gel electrophoresis and western blotting.

**Cell proliferation assays.** Cell proliferation was measured using a 3-(4,5-dime-thylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Ten thousand MDA-MB-231 cells or 1000 4T1 cells per well (four wells per sample) were seeded in 96-well plates with 10% FBS. The cell proliferation rate was examined by photometric analysis using a microplate reader (BioTek); calculate the signal sample as OD570 minus OD650.

**Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)** Total RNA in cancer cells was extracted using TRIzol (Life Technologies) and then reverse-transcribed using an iScript cDNA Synthesis Kit (Bio-Rad; #1708899). Quantitative reverse transcription (qRT)-polymerase chain reaction (PCR) analysis of mRNA expression was performed using SYBR Fast Universal qPCR Master Mix (Kapa Biosystems; #KK4602) with a StepOnePlus real-time PCR system (Applied Biosystems). Relative mRNA expression was calculated using the 2**^-ΔΔCt^ method with logarithmic transformation. SYBR primers were purchased from Sigma or Integrated DNA Technologies (IDT). The following primers were used: human PTHLH (encoding PTHrP): F5'-TTTACCGGGACAGATCTTTCC-3', R5'-TTTCTTCCCAAGGTTCTTGAG-3'; mouse PTHrP (encoding PTHrP): F5'-CATCAGCTGATCTGAAAGC-3', R5'-GGTTGTTTTGTTGGTGGGAG-3'; human inter leukin-8 (IL-8; CXCL8): F5'-GAGTGATGAGTGAGTGAGGCTACCC-3', R5'-AGACAGAGCCTTTCTTACATGAAA-3'; mouse IL-8 (Cxcl8): F5'-TTCTTCTGCTGCTGCTCTAAAC-3', R5'-ACTGCTATCTCCTTCTTCTGTA-3'; human ACTB: F5'-CATGATGTTGAGATCTACCCAGG-3', R5'-CTCTAACTGTGCAAACTCTGATATT-3'; mouse ACTB (encoding ACTB): F5'-GCACCTTCAAGGCAAGAAGG-3', R5'-AGGCTGATGCTTCTGGTGTGG-3'; human ITGB1 (encoding ITGB1): F5'-TTCTCGCAGCCATCTGCTAT-3', R5'-AGCAGGACGTCTTCTCAGTCAAAAA-3'; mouse ITGB3 (encoding ITGB3): F5'-CTGGATAACTTCTCTGAGGAGG-3', R5'-CTGGATAACTTCTCTGAGGAGG-3'; E. coli primers AGATTATTATGTATGATACACCATGCC-3' and R5'-TTTAAATGTTGGTGCCCTC-3'.
**Triple co-culture assay and TRAP staining.** Triple co-culture assay and TRAP staining were performed as described previously34. Murine RAW 264.7 pre-osteoclasts (3 × 10^6 cells/well) were seeded directly into the wells of six-well culture plates, and MC3T3 cells (3 × 10^5 cells/well) were seeded into Millicell Hanging Cell Culture Inserts (Millipore) in the six-well co-culture plates. The next day, MC3T3 cells were allowed to attach to the membranes of the inserts, and luciferase/green fluorescent protein (GFP)-labeled (GFP+) MDA-MB-231, 231.sgCtrl, 231.KO1, and 231.KO2 cells (3 × 10^5 cells/well) were seeded on top of the MC3T3 cell layer in triplicate and treated with 5 ng/ml TGFiβ, 2 μM GSK126, or a vehicle. Co-culture assays were performed in Dulbecco’s modified Eagle’s medium/high glucose medium supplemented with 10% FBS and that was changed every 2 days. TRAP staining of osteoclasts was performed on day 6 using a leukocyte acid phosphatase kit (Sigma-Aldrich; #387 A). TRAP+ multinucleated cells were scored as mature osteoclasts and quantified. MC3T3 cells and GFP+ tumor cells were trypsinized from the inserts and calculated GFP+ cell numbers using flow cytometry.

**Luciferase reporter assay.** Luciferase reporter assay was performed as described previously34; pGL4.10 (Luc2; E665A) was purchased from Promega. The pGL4.10-ITGB1 reporter and a control Renilla luciferase vector were co-transfected into breast cancer cell lines using Lipofectamine 3000 transfection kit (Invitrogen; #3000-008). After 48 h, luciferase activity was measured using a Dual-Luciferase Reporter Assay kit (Promega; E1910) with a Victor Lumitrac (Turner Biosystems). The ITGB1 promoter was generated via amplification of a genomic DNA sequence with PCR using the designed primers and then inserted upstream of the luciferase reporter gene in the pGL4.10 vector. The primer sequences for the ITGB1 reporter were F5- CAGGATCAGCTTCC-3’. All fold-enrichment values were normalized according to those of IgG. HOX/A9 was used as a positive control for EZH2 and H3K27me3 binding.

**Mass spectrometry.** Liquid chromatography/tandem-mass spectrometry was used to identify phosphorylation sites of TGFR1. HEK 293FT cells were transfected with pRK5-TGFR1-FLAG plasmid (Addgene, #14833) using Lipofectamine 3000 transfection kit; HEK 293FT cell lysates were immunoprecipitated with an antibody. After protein gel electrophoresis, TGFR1 band was excised from the gel and digested with trypsin. The resulting peptides were analyzed by LC/MS/MS (Applied Biosystems QSTAR Elite, Applied Biosystems QTRAP 3500, and Applied Biosystems QTRAP 6500) to determine their sequence with PCR using the designed primers and then inserted upstream of the luciferase reporter gene in the pGL4.10 vector. The primer sequences for the ITGB1 reporter were F5- CAGGATCAGCTTCC-3’. All fold-enrichment values were normalized according to those of IgG. HOX/A9 was used as a positive control for EZH2 and H3K27me3 binding.

**Migration and invasion assays.** For a migration assay, breast cancer cells (30,000 cells/well) in FBS-free medium were placed on the top side of the uncoated transwell inserts. All sample sizes were listed in the corresponding presentations in this manuscript were generated and displayed with PyMOL.

**Animal experiments.** All animal experiments were carried out in accordance with protocols (00001397-RN02) approved by the MD Anderson Institutional Animal Care and Use Committee. The study is compliant with all relevant ethical reg- ulations regarding animal research. Aethion Ncr nu/nu mice (strain: 4009218) were obtained from Jackson Lab. The mice were exposed to a 12-h light/12-h dark cycle at 22–24 °C with 50–60% humidity, bred as specific pathogen-free animals, and given free access to food and water. The number of mice used in each experimental group was determined via power analysis or based on prior experience with similar animal models, and results were presented as mean ± SD. All sample sizes were listed in the corresponding figure legend or figures. All mice used were the same age (8 weeks) and had similar body weights. Two different injection models were used for bone metastasis studies. (1) Intracardiac injection model A: 1 × 10^5 cells of the 231–1566 sublines or E2H12 knockdown MDA-MB-231 sublines were injected into the left ventricle in anesthetized femoral artery. Ncr nu/nu mice. GSK126 was dissolved in 20% cyclodextrin (Captoril, CyDex Pharma- ceuticals) and adjusted to a pH level of 4.0 to 4.5 with 1 N acetic acid following the instructions described by McCabe et al.39. GSK126 was administered to the mice via intraperitoneal (i.p.) injection three times a week at a dose of 150 mg/kg after each cardiac injection. (2) Intraperitoneal injection model: Groups of 6- to 8-week-old female athymic Ncr nu/nu mice were injected with 1566.KO. The complex was then visualized and analyzed with PyMOL. Two final models were selected on the basis of substrate recognition and phosphorylation mechanism from ATP hydrolysis to TGFR1.

**TGFR1-TGFR2 complex and Integrin-β1-TGFR1 complex modeling by molecular docking.** High-resolution crystal structures of the cytoplasmic domain of TGFR1 (PDB ID: 1ias) and the kinase domain of TGFR2 (AMPPNN, an ATP analog, bound state, PDB ID: 5e92) were obtained from Protein Data Bank. A pharmacophore group was added to the kinase domain of TGFR2 using PyTMs66. The complex was then visualized and analyzed with PyMOL. Two final models were selected on the basis of substrate recognition and phosphorylation mechanism from ATP hydrolysis to TGFR1.

**Animal experiments.** All animal experiments were carried out in accordance with protocols (00001397-RN02) approved by the MD Anderson Institutional Animal Care and Use Committee. The study is compliant with all relevant ethical reg- ulations regarding animal research. Aethion Ncr nu/nu mice (strain: 4009218) were obtained from Jackson Lab. The mice were exposed to a 12-h light/12-h dark cycle at 22–24 °C with 50–60% humidity, bred as specific pathogen-free animals, and given free access to food and water. The number of mice used in each experimental group was determined via power analysis or based on prior experience with similar animal models, and results were presented as mean ± SD. All sample sizes were listed in the corresponding figure legend or figures. All mice used were the same age (8 weeks) and had similar body weights. Two different injection models were used for bone metastasis studies. (1) Intracardiac injection model A: 1 × 10^5 cells of the 231–1566 sublines or E2H12 knockdown MDA-MB-231 sublines were injected into the left ventricle in anesthetized femoral artery. Ncr nu/nu mice. GSK126 was dissolved in 20% cyclodextrin (Captoril, CyDex Pharma- ceuticals) and adjusted to a pH level of 4.0 to 4.5 with 1 N acetic acid following the instructions described by McCabe et al.39. GSK126 was administered to the mice via intraperitoneal (i.p.) injection three times a week at a dose of 150 mg/kg after each cardiac injection. (2) Intraperitoneal injection model: Groups of 6- to 8-week-old female athymic Ncr nu/nu mice were injected with 1566.KO. The complex was then visualized and analyzed with PyMOL. Two final models were selected on the basis of substrate recognition and phosphorylation mechanism from ATP hydrolysis to TGFR1.

**TGFR1-TGFR2 complex and Integrin-β1-TGFR1 complex modeling by molecular docking.** High-resolution crystal structures of the cytoplasmic domain of TGFR1 (PDB ID: 1ias) and the kinase domain of TGFR2 (AMPPNN, an ATP analog, bound state, PDB ID: 5e92) were obtained from Protein Data Bank. A pharmacophore group was added to the kinase domain of TGFR2 using PyTMs66. The complex was then visualized and analyzed with PyMOL. Two final models were selected on the basis of substrate recognition and phosphorylation mechanism from ATP hydrolysis to TGFR1.

**Animal experiments.** All animal experiments were carried out in accordance with protocols (00001397-RN02) approved by the MD Anderson Institutional Animal Care and Use Committee. The study is compliant with all relevant ethical reg- ulations regarding animal research. Aethion Ncr nu/nu mice (strain: 4009218) were obtained from Jackson Lab. The mice were exposed to a 12-h light/12-h dark cycle at 22–24 °C with 50–60% humidity, bred as specific pathogen-free animals, and given free access to food and water. The number of mice used in each experimental group was determined via power analysis or based on prior experience with similar animal models, and results were presented as mean ± SD. All sample sizes were listed in the corresponding figure legend or figures. All mice used were the same age (8 weeks) and had similar body weights. Two different injection models were used for bone metastasis studies. (1) Intracardiac injection model A: 1 × 10^5 cells of the 231–1566 sublines or E2H12 knockdown MDA-MB-231 sublines were injected into the left ventricle in anesthetized femoral artery. Ncr nu/nu mice. GSK126 was dissolved in 20% cyclodextrin (Captoril, CyDex Pharma- ceuticals) and adjusted to a pH level of 4.0 to 4.5 with 1 N acetic acid following the instructions described by McCabe et al.39. GSK126 was administered to the mice via intraperitoneal (i.p.) injection three times a week at a dose of 150 mg/kg after each cardiac injection. (2) Intraperitoneal injection model: Groups of 6- to 8-week-old female athymic Ncr nu/nu mice were injected with 1566.KO. The complex was then visualized and analyzed with PyMOL. Two final models were selected on the basis of substrate recognition and phosphorylation mechanism from ATP hydrolysis to TGFR1.
distress. After anesthetized mice were intraperitoneally injected with 75 mg/kg D-Luciferin, BLI was performed using a Xenogen IVIS 200 imaging system (PerkinElmer). Analysis of bone metastasis was performed using living image software by measuring the photon flux in the hindlimbs of mice. The photon flux curves were normalized according to the signal on the day when mice were given the drug GSK126 or vehicle. Bone metastasis-free survival curves showed the time point at which each mouse experienced bone metastasis development according to threshold BLI signals in the hindlimbs. X-ray images of hindlimbs of mice were obtained using an IVIS Lumina XR system (PerkinElmer).

**Immunohistochemistry staining and scoring system.** Standard immunohistochemistry (IHC) staining was performed as described previously. The immunoreactive score (IRS) was used to quantify the IHC staining, ranging from 0 to 12 (0 = absent, 1–2 = weak, 3 = moderate, 4–8 = strong, 8 = very strong). The IRS was independently evaluated by two pathologists blinded to the experimental groups. All quantitative experiments were performed using at least three independent biological repeats, and the results are presented as means ± standard deviation (S.D.) or means ± standard error of the mean (S.E.M.). One-way analysis of variance (multiple groups) or t-tests (two groups) were used to compare the means for two or more samples using the Prism 8 software program (GraphPad Software). Survival was analyzed using Kaplan–Meier curves and log–rank tests. P-values < 0.05 (two-sided) were considered statistically significant. For IHC score, ten visual fields from different areas of each tumor were evaluated by two pathologists independently (blinded to experiment groups). For migration, invasion, and TRAP+ osteoclasts staining experiments, more than three visual fields were evaluated, and three independent biology repeats were performed. Real-time images of micrographs were shown to represent reproducible data from various experiments using micrographs.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**References**

1. Siegel, R. L., Miller, K. D. & Jemal, A. Cancer statistics, 2019. CA Cancer J. Clin. 69, 7–34 (2019).
2. Fornetti, J., Welm, A. L. & Stewart, S. A. Understanding the bone in cancer metastasis. J. Bone Min. Res. 33, 2099–2113 (2018).
3. Rossi, L., Longhittano, C., Kola, F. & Del Grande, M. State of art and advances on the treatment of bone metastases from breast cancer: a concise review. Chin. Clin. Oncol. 9, 18 (2020).
4. Mundy, G. R. Metastasis to bone: causes, consequences and therapeutic opportunities. Nat. Rev. Cancer 2, 584–593 (2002).
5. Zhang, W., Bado, I., Wang, H., Lo, H. C. & Zhang, X. H. Bone metastasis: find your niche and your niche and your niche. Nature 509, 631–635 (2014).
6. Nakao, A. et al. Identification of Smad7, a TGF-beta-inducible antagonist of TGF-beta signalling. Nature 389, 631–635 (1997).
7. Heldin, C. H. & Moustakas, A. Signaling receptors for TGF-beta family members. Cold Spring Harb. Perspect. Biol. https://doi.org/10.1101/cshperspect.a022533 (2016).
8. Wieser, R., Warna, J. L. & Massague, J. GS domain mutations that constitutively activate T beta R-I, the downstream signaling component in the TGF-beta receptor complex. EMBO J. 14, 2199–2208 (1995).
9. Wieser, R., Warna, J. L. & Massague, J. G5 domain mutations that constitutively activate T beta R-I, the downstream signaling component in the TGF-beta receptor complex. EMBO J. 14, 2199–2208 (1995).
10. Naik, A. et al. Neutrophil-1 promotes the oncogetic Tenascin-C/integrin beta3 pathway and modulates chemoresistance in breast cancer cells. BMC Cancer 18, 533 (2018).
11. Zhang, L. et al. Blocking immunosuppressive mechanisms of pY696–EZH2-driven brain metastases. Sci. Transl. Med. 12, eaae5387 (2020).
12. Cao, R. & Zhang, Y. SUZ12 is required forboth the histone methyltransferase activity and the silencing function of the EED–EZH2 complex. Mol. Cell 15, 57–67 (2004).
13. Campbell, M. G. et al. Cryo-EM reveals integrin-mediated TGF-beta activation without release from latent TGF-beta. Cell 180, 490–501 e16 (2020).
14. Nagae, M. et al. Crystal structure of alpha3beta1 integrin ectodomain: atomic details of the fibronectin receptor. J. Cell Biol. 197, 131–140 (2012).
15. Sulam, J., P. J., Jean, C. & Schlaepfer, D. D. FAK in cancer: mechanistic findings and clinical applications. Nat. Rev. Cancer 14, 598–610 (2014).
16. Stockis, J. et al. Blocking immunosuppression by human Tregs in vivo with antibodies targeting integrin alphaVbeta8. Proc. Natl. Acad. Sci. USA 114, E10161–E10168 (2017).
17. Nogues, J. S. et al. The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. Cell 96, 319–328 (1999).
41. Feldkoren, B., Hutchinson, R., Rapoport, Y., Mahajan, A. & Margulis, V. Integrin signaling potentiates transforming growth factor-beta 1 (TGF-beta1) dependent down-regulation of E-Cadherin expression—Important implications for epithelial to mesenchymal transition (EMT) in renal cell carcinoma. Exp. Cell Res. 355, 57–66 (2017).

42. Cai, T., Lei, Q. Y., Wang, L. Y. & Zha, X. L. TGF-beta 1 modulated the expression of alpha 5 beta 1 integrin and integrin-mediated signaling in human hepatocellular carcinoma cells. Biochem Biophys. Res. Commun. 274, 519–525 (2000).

43. Chen, Y. et al. Focal adhesion kinase promotes hepatic stellate cell activation by regulating plasma membrane localization of TGFbeta receptor 2. Hepatol. Commun. 4, 268–283 (2020).

44. Wendt, M. K. & Schiemann, W. P. Therapeutic targeting of the focal adhesion complex predicts oncogenic TGF-beta signaling and metastasis. Breast Cancer Res 11, R68 (2009).

45. Souchelnytskyi, S., ten Dijke, P., Miyazono, K. & Heldin, C. H. Phosphorylation of Ser165 in TGF-beta type 1 receptor modulates TGF-beta 1-induced cellular responses. EMBO J. 15, 6231–6240 (1996).

46. van Dinther, M. et al. ALK2 R206H mutation linked to chondrodysplasia ossificans progressiva confers constitutive activity to the BMP type 1 receptor and sensitizes mesenchymal cells to BMP-induced osteoblast differentiation and bone formation. J. Bone Min. Res. 25, 1208–1215 (2010).

47. Massague, J., Seoane, J. & Wotton, D. Smad transcription factors. Genes Dev. 19, 2783–2810 (2005).

48. He, A. et al. PRCC directly methylates GATA4 and represses its transcriptional activity. Genes Dev. 26, 37–42 (2012).

49. Sanulli, S. et al. Jarid2 methylation via the PRC2 complex regulates histone H3K27me3 deposition during cell differentiation. Mol. Cell 57, 769–783 (2015).

50. Lee, J. M. et al. EZH2 generates a methyl degron that is recognized by the DCAF1/DDB1/CUL4 E3 ubiquitin ligase complex. Mol. Cell 77, 363–376 (2019).

51. Vasanthakumar, A. et al. A non-canonical function of Ezh2 preserves immune homeostasis. EMBO Rep. 18, 619–631 (2017).

52. Gonzalez, M. E. et al. Histone methyltransferase EZH2 induces Akt-dependent genomic instability and BRCA1 inhibition in breast cancer. Cancer Res. 71, 2360–2370 (2011).

53. Xu, K. et al. EZH2 oncogenic activity in castration-resistant prostate cancer cells is Polycym-blood-dependent. Science 338, 1465–1469 (2012).

54. Kim, J. et al. Polycomb- and methylation-independent roles of EZH2 as a transcription activator. Cell Rep. 25, 2808–2820 e2804 (2018).

55. Gulati, N., Beguelin, W. & Giulino-Roth, L. Enhancer of zeste homolog 2 (EZH2) inhibitors. Leuk. Lymphoma 59, 1574–1585 (2018).

56. McCabe, M. T. et al. EZH2 inhibition as a therapeutic strategy for lymphoma with EZH2-activating mutations. Nature 492, 108–112 (2012).

57. Kondo, Y. Targeting histone methyltransferase EZH2 as cancer treatment. J. Biochem. 156, 249–257 (2014).

58. Italiano, A. et al. Tazemetostat, an EZH2 inhibitor, in relapsed or refractory B-cell non-Hodgkin lymphoma and advanced solid tumours: a first-in-human, open-label, phase 1 study. Lancet Oncol. 19, 649–659 (2018).

59. Hirukawa, A. et al. Targeting EZH2 reactivates a breast cancer subtype-specific anti-metastatic transcriptional program. Nat. Commun. 9, 2547 (2018).

60. Yonts, S. et al. Inhibition of EZH2 catalytic activity selectively targets a metastatic subpopulation in triple-negative breast cancer. Cell Rep. 30, 755–770 e756 (2020).

61. Wang, H. et al. Bone-in-culture array as a platform to model early-stage bone metastases and discover anti-metastasis therapies. Nat. Commun. 8, 15045 (2017).

62. Khotskaya, Y. B. et al. S6K1 promotes invasiveness of breast cancer cells in a model of metastasis of triple-negative breast cancer. Am. J. Transl. Res. 6, 361–376 (2014).

63. Ran, F. A. et al. Genome engineering using the CRISPR-Cas9 system. Nat. Protoc. 8, 2281–2308 (2013).

64. Zhang, S. et al. SRC family kinases as novel therapeutic targets to treat breast cancer brain metastases. Cancer Res. 73, 5764–5774 (2013).

65. Hu, J. et al. Non-parametric quantification of protein lysate arrays. Bioinformatics 23, 1986–1994 (2007).

66. Warnecke, A., Sandalova, T., Achiour, A. & Harris, R. A. PyTMs: a useful PyMOL plugin for modeling common post-translational modifications. BMC Bioinformatics 15, 370 (2014).

67. Kozak, D. et al. The ClusPro web server for protein–protein docking. Nat. Protoc. 12, 255–278 (2017).

68. Lu, J. et al. 14-3-3zeta Cooperates with ErbB2 to promote ductal carcinoma in situ progression to invasive breast cancer by inducing epithelial–mesenchymal transition. Cancer Cell 16, 195–207 (2009).

Acknowledgements
We thank members of the Yu’s laboratory for insightful discussions. We thank D. Norwood, the Department of Scientific Publications of MD Anderson Cancer Center for article revision. This work was supported by National Institutes of Health (NIH) grants R01CA184836 (D.Y.), R01CA208213 (D.Y.), and R01CA231149 (D.Y.), the METAvivor grants 56675 and 58284 (D.Y.), and NIH Cancer Center Support Grant P30CA016672 to MD Anderson Cancer Center (Functional Genomics Core, Flow Cytometry and Cellular Imaging resource, Advanced Technology Genomics Core, Research Histology Core Laboratory, Cytogenetics and Cell Authentication Core, Functional Proteomics Reverse Phase Protein Array Core, and Research Animal Support Facility-Houston). J.Q. received fellowship from China Scholarship Council 201706280072. Y.-W.H. received fellowship from the Ministry of Education, Taiwan: The International Co-cultivation of Talent Program. D.Y. is the Hubert L. and Olive Stringer Distinguished Chair in Basic Science at MD Anderson. Mass spectrum is performed at and supported in part by the Clinical and Translational Proteomics Service Center at the University of Texas Health Science Center.

Author contributions
L.Z., J.Q., and D.Y. developed original hypothesis and designed experiments. L.Z., J.Q., Y.Q., Y.D., Y.-W.H., Z.Z., P.L., J.Y., B.H., S.Z., and D.Y. performed experiments and/or analyzed data. L.Z., J.Q., and D.Y. wrote and edited the manuscript. D.Y. supervised and provided resources for the study.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-30105-0.

Correspondence and requests for materials should be addressed to Dihua Yu.

Peer review information Nature Communications thanks Kohei Miyazono, Johanna Iwasa, and the other, anonymous, reviewers for their contribution to the peer review of this work.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access
This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022