ATM-mediated stabilization of ZEB1 promotes DNA damage response and radioresistance through CHK1

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Epithelial–mesenchymal transition (EMT) is associated with characteristics of breast cancer stem cells, including chemoresistance and radioresistance. However, it is unclear whether EMT itself or specific EMT regulators play causal roles in these properties. Here we identify an ATM-inducing transcription factor, zinc finger E-box binding homeobox 1 (ZEB1), as a regulator of radiosensitivity and DNA damage response. Radioresistant subpopulations of breast cancer cells derived from ionizing radiation exhibit hyperactivation of the kinase ATM and upregulation of ZEB1, and the latter promotes tumour cell radioresistance in vitro and in vivo. Mechanistically, ATM phosphorylates and stabilizes ZEB1 in response to DNA damage, ZEB1 in turn directly interacts with USP7 and enhances its ability to deubiquitylate and stabilize CHK1, thereby promoting homologous recombination-dependent DNA repair and resistance to radiation. These findings identify ZEB1 as an ATM substrate linking ATM to CHK1 and the mechanism underlying the association between EMT and radioresistance.

Radiation therapy causes cell death by inducing single- and double-strand DNA breaks¹². The rationale for treating tumour tissues with radiation without damaging normal tissues is that compared with normal cells, tumour cells are actively dividing and often have defects in DNA damage repair machinery, and thus are less able to repair DNA damage¹. A major cause of failure in radiation treatment is intrinsic and therapy-induced radioresistant tumour cells, which exhibit increased DNA repair ability⁴.

The DNA damage response (DDR) pathway consists of sensors, transducers and effectors⁸⁹. In response to genotoxic damage, the RAD9–HUS1–RAD1 (9–1–1) complex is recruited to the DNA damage sites by a RAD17-containing protein complex and then facilitates ATR-mediated phosphorylation and activation of CHK1, an effector protein kinase that regulates S phase progression and G2/M cell cycle arrest⁵⁷. Another sensor complex, the MRE11–RAD50–NBS1 (MRN) complex, detects double-strand breaks (DSBs), recruits ATM and promotes ATM-mediated phosphorylation of histone H2AX (γH2AX) surrounding the DNA breaks⁸⁹. Subsequently, a number of signalling and repair proteins accumulate at DNA lesions and form discrete foci¹⁰¹².

Recently, cancer stem cells have been shown to promote radioresistance through activation of DDR (refs 13,14). Moreover, the EMT trans-differentiation program can generate cells with stem-like properties¹⁵. EMT can be induced by various transcription factors, including Twist, Snail, Slug, ZEB1 and ZEB2 (refs 16,17). However, it is unclear whether EMT itself or specific EMT regulators cause properties associated with cancer stem cells such as chemoresistance and radioresistance.

Here, we found that the EMT regulator ZEB1 promotes DDR and tumour radioresistance. This regulation is initiated by phosphorylation and stabilization of ZEB1 by ATM and is mediated by stabilization of CHK1 by a ZEB1-interacting deubiquitylase, USP7.
ZEB1 underlies the association between EMT and radioresistance

To examine the association between EMT and radioresistance, we overexpressed Snail, Twist or ZEB1 in the experimentally immortalized, non-transformed human mammary epithelial cells, termed HMLE cells. Each of these transcription factors induced EMT—as evidenced by changes in morphology (Supplementary Fig. 1a), downregulation of E-cadherin and upregulation of vimentin (Fig. 1a), and increased clonogenic survival on irradiation (Fig. 1b and Supplementary Fig. 1b). In each case, expression of Snail, Twist and ZEB1 was upregulated; in particular, overexpression of either Snail or Twist increased ZEB1 expression to a level as high as that of ZEB1-overexpressing cells (Fig. 1a). Next, we silenced each of the
ZEB1 is upregulated in radioresistant cancer cells and promotes tumour radioresistance. (a) Schematic representation of generation of a radioresistant subline (SUM159-P2) from parental SUM159 cells (SUM159-P0). (b) Clonogenic survival assays of SUM159-P0 and SUM159-P2 cells. IR, ionizing radiation. n = 3 wells per group. (c) Immunoblotting of γH2AX and HSP90 in SUM159-P0 and SUM159-P2 cells treated with 6 Gy ionizing radiation. (d) Immunoblotting of Snail, Twist, ZEB1 and GAPDH in SUM159-P0 and SUM159-P2 cells. SUM159-P0 cells transfected with Snail or Twist were used as positive controls. (e) Immunoblotting of Snail, Twist, ZEB1 and GAPDH in SUM159-P0 cells transfected with Snail, Twist or ZEB1. (f) Clonogenic survival assays of SUM159-P0 cells transfected with Snail, Twist or ZEB1. n = 3 wells per group. Significance of Mock versus ZEB1 is shown. (g) Clonogenic survival assays of SUM159-P2 cells transduced with ZEB1 shRNA. Inset: immunoblotting of ZEB1 and GAPDH. n = 3 wells per group. (h,i) Tumour size of mice bearing control (scramble) or ZEB1 shRNA-transduced SUM159-P2 xenografts. Tumours were locally irradiated with 15 Gy single dose (h) or 2 Gy fractionated dose (XRT) twice per day for 7 consecutive days (i). n = 5 mice per group. General linear model multivariate analysis was performed to determine statistical significance. (j) Immunoblotting of ZEB1 and HSP90 in tumour lysates. Data in b,f,i are the mean of biological replicates from a representative experiment, and error bars indicate s.e.m. Statistical significance in b, f and g was determined by a two-tailed, unpaired Student’s t-test. The experiments were repeated 3 times. For source data, see Supplementary Table 3. Uncropped images of blots are shown in Supplementary Fig. 7.

To determine whether ZEB1 is indeed upregulated in radioresistant tumour cells, we employed γ-ionizing radiation to select the radioresistant subpopulation from the SUM159 human breast cancer cells that express moderate levels of ZEB1. After a 6 Gray (Gy) dose, surviving cells formed colonies. We pooled the colonies and repeated the dose one more time (Fig. 2a). Cells derived from this selection, named SUM159-P2 cells, exhibited increased clonogenic survival on irradiation compared with the parental SUM159 cells (SUM159-P0; Fig. 2b). Irradiation causes DSBs resulting in the formation of γH2AX foci, and the persistence of γH2AX foci marks delayed repair and correlates with radiosensitivity21-23. At 24 h after irradiation, γH2AX remained in SUM159-P0 cells but disappeared in SUM159-P2 cells (Fig. 2c), indicating that this radioresistant subline has enhanced clearance of DNA breaks.

Next, we examined the protein levels of Snail, Twist and ZEB1. Only one factor, ZEB1, was significantly upregulated in SUM159-P2 three transcription factors in HMLE cells overexpressing Snail, Twist or ZEB1, which did not cause reversal of EMT (Fig. 1c). Notably, only knockdown of ZEB1 reduced radioresistance (Fig. 1d), suggesting that ZEB1 underlies the association between EMT and radioresistance. Consistent with this notion, we observed upregulation of ZEB1 in the survival fraction of mock-infected HMLE cells (Supplementary Fig. 1c); moreover, the survival fraction of ZEB1-depleted HMLE cells re-expressed ZEB1 (Supplementary Fig. 1c).

We then overexpressed these three transcription factors in the MCF7 human breast cancer cell line. Unlike HMLE cells, MCF7 cells express intact p53, which acts as a barrier to EMT induction19,20. Indeed, none of these three transcription factors induced EMT in MCF7 cells (Fig. 1e and data not shown). Moreover, only ZEB1, but not Snail or Twist, conferred radioresistance on these cells (Fig. 1f). Taken together, it may not be EMT itself that causes radioresistance; instead, it is a specific EMT regulator, ZEB1, that plays a causal role in regulating the response to radiation.
Figure 3 ZEB1 regulates DNA damage repair. (a) γH2AX and DAPI staining of SUM159-P2 cells transduced with ZEB1 shRNA, 24 h after 6 Gy ionizing radiation. Scale bar, 10 μm. (b) Immunoblotting of ZEB1, γH2AX, H2AX and GAPDH in SUM159-P2 cells transduced with ZEB1 shRNA, at the indicated time points after 6 Gy ionizing radiation (IR). (c,d) Images (c) and data quantification (d) of comet assays of SUM159-P2 cells transduced with ZEB1 shRNA, at the indicated time points after 6 Gy ionizing radiation. n = 62 cells per group. Scale bar, 50 μm (c). (e) Immunoblotting of ZEB1 and GAPDH in U2OS_DR-GFP cells transfected with ZEB1 siRNA alone or in combination with ZEB1. (f) Homologous recombination repair assays of U2OS_DR-GFP cells transfected with ZEB1 siRNA alone or in combination with ZEB1. n = 3 wells per group. Data in d and f are the mean of biological replicates from a representative experiment, and error bars indicate s.e.m. Statistical significance was determined by a two-tailed, unpaired Student’s t-test. The experiments were repeated 3 times. For source data, see Supplementary Table 3. Uncropped images of blots are shown in Supplementary Fig. 7.
(shRNA) was retained throughout this tumour radiosensitivity study (Fig. 2j). Taken together, ZEB1 is required for the radioresistance of these breast cancer cells in vitro and in vivo.

ZEB1 regulates DNA damage repair

After ionizing radiation treatment, γH2AX foci persist longer in radiosensitive cell lines than in radioresistant lines26. In ZEB1 shRNA-expressing SUM159-P2 cells but not cells infected with a scrambled control, we observed persistence of γH2AX foci 24 h after ionizing radiation treatment (Fig. 3a,b), indicating that ZEB1-depleted cells were less able to repair DNA lesions. To directly gauge damaged DNA, we performed a comet assay to detect both single- and double-strand DNA breaks. At 24 h after ionizing radiation treatment, ZEB1-depleted SUM159-P2 cells exhibited a 4.5-fold increase in the comet 'tail moment' (the percentage of the DNA in the tail × the length of the tail in micrometres)—a previously described measure of DNA damage26, compared with the control cells (Fig. 3c,d).

Our results demonstrate that ZEB1 is required for DSB clearance. In mammalian cells, a key conserved pathway involved in DSB repair is the homologous recombination pathway25. To determine the effect of ZEB1 on homologous recombination repair, we used a U2OS cell clone with chromosomal integration of an homologous recombination repair reporter consisting of two differentially mutated GFP genes (SceGFP and iGFP) oriented as direct repeats (DR-GFP); in this assay, expression of I-SceI endonuclease generates a site-specific DSB in the SceGFP coding region, and when this DSB is repaired by homologous recombination, the expression of GFP is restored and can be analysed by flow cytometry to gauge the efficiency of homologous recombination repair26,27. We found that on I-SceI expression, ZEB1-depleted U2OS cells exhibited a significant decrease (~50%) in the percentage of GFP-positive cells, indicating defective homologous recombination repair (Fig. 3e,f). Moreover, re-expression of ZEB1 in ZEB1 short interfering RNA (siRNA)-expressing U2OS cells restored homologous-recombination-based repair (Fig. 3e,f). Collectively, these results suggest that ZEB1 is required for homologous-recombination-mediated DNA damage repair and the clearance of DNA breaks.

ZEB1 regulates radiosensitivity through USP7-mediated stabilization of CHK1

We reasoned that ZEB1 regulates radiosensitivity by modulating DDR pathways. CHK1 and CHK2 are two critical effector kinases in DDR and checkpoint control24–30, which prompted us to examine their status in ZEB1-depleted breast cancer cells. Interestingly, knockdown of ZEB1 in SUM159-P2 cells resulted in a significant reduction in CHK1 protein levels in the presence or absence of ionizing radiation (Fig. 4a); in contrast, neither the CHK2 total protein level nor its phosphorylation was affected (Fig. 4a). Moreover, expression of an RNA-mediated interference (RNAi)-resistant ZEB1 mutant completely reversed the effect of ZEB1 shRNA on CHK1, γH2AX and clonogenic survival (Supplementary Fig. 3a,b). Conversely, overexpression of ZEB1 in MCF7 cells significantly upregulated CHK1 and promoted the clearance of DNA breaks after ionizing radiation treatment (gauged by γH2AX; Fig. 4b). In addition, Zeb1-deficient mouse embryonic fibroblasts (MEFs) exhibited downregulation of Chk1 (Fig. 4c).

CHK1 activates the G2 checkpoint in response to stalled replication forks or DNA damage31. As anticipated, irradiation resulted in the arrest of SUM159-P2 cells in the G2/M phase, and knockdown of ZEB1 led to a moderate but significant decrease in the G2/M population (Supplementary Fig. 3c). CHK1 levels are known to vary depending on the cell cycle phase32. To exclude the indirect effect due to the difference in cell cycle, we synchronized scramble-transfected or ZEB1 siRNA-transfected SUM159-P2 cells in the G2/M phase by nocodazole treatment and released the cells at different time points. As expected, CHK1 was detected in S and G2/M phases (Fig. 4d). We found that in these synchronized cells, CHK1 levels correlated with ZEB1 levels, and that knockdown of ZEB1 led to downregulation of CHK1 at each cell cycle stage (Fig. 4d), which suggested that the downregulation of CHK1 caused by ZEB1 depletion is not an indirect effect of cell cycle changes.

We assessed the effect of CHK1 on radiosensitivity. Silencing CHK1 expression recapitulated the effect of ZEB1 shRNA on sensitizing SUM159-P2 cells to ionizing radiation (Fig. 4e). Conversely, re-expression of CHK1 in ZEB1-depleted SUM159-P2 cells rescued radiosensitivity (Fig. 4f,g). Moreover, knockdown of CHK1 reversed ZEB1-induced radiosensitivity in SUM159-P0 cells (Fig. 4h,i). These data suggest that ZEB1 regulates tumour cell radiosensitivity through, at least in part, CHK1.

As depletion of ZEB1 downregulated CHK1 protein (Fig. 4a) but not CHK1 mRNA (Supplementary Fig. 4a), and because CHK1 is subject to ubiquitin-dependent degradation following replication stress33–35, we reasoned that ZEB1 may regulate CHK1 protein levels through ubiquitin-dependent mechanisms. Indeed, knockdown of ZEB1 significantly induced the polyubiquitination of endogenous CHK1 protein with or without ionizing radiation (Fig. 5a).

To further investigate the mechanism by which ZEB1 regulates CHK1 ubiquitylation, we attempted to identify ZEB1-interacting proteins using a triple-epitope (S-protein, FLAG tag and streptavidin-binding peptide)-tagged version of ZEB1 (SFB–ZEB1). Tandem-affinity purification using streptavidin–Sepharose beads and S-protein–agarose beads followed by mass spectrometric analysis identified several reported ZEB1 interactors including CTBP2, CTBP1 and SIRT1 (refs 36–38, as well as a previously undescribed ZEB1 interactor, USP7 (Supplementary Table 1 and Fig. 5b).

USP7 is a deubiquitylating enzyme with several reported substrates, such as p53 (ref. 39), Mdm2 (refs 40,41), HLTF (ref. 42), PTEN (ref. 43) and Claspin44. Co-immunoprecipitation assays confirmed that both USP7 and CHK1 could be detected in ZEB1 immunoprecipitates (Fig. 5c), and that both ZEB1 and CHK1 were present in USP7 immunoprecipitates (Fig. 5d). Moreover, purified GST–USP7 could bind to purified MBP–tagged ZEB1 under cell-free conditions (Fig. 5e), demonstrating direct interaction between ZEB1 and USP7.

To investigate whether USP7 regulates the stability of CHK1 protein, we examined CHK1 protein levels in the presence of cycloheximide (CHX), an inhibitor of translation. Notably, overexpression of USP7 in 293T cells led to a pronounced increase in CHK1 protein stability (Fig. 5f and Supplementary Fig. 4b). Conversely, knockdown of USP7 in SUM159-P2 cells reduced CHK1 stability (Fig. 5g and Supplementary Fig. 4c) but not ZEB1 stability (Supplementary Fig. 4d). Interestingly, knockdown of ZEB1 in SUM159-P2 cells destabilized CHK1, but not other
USP7 substrates such as HLTF, p53 or Claspin (Fig. 5h and Supplementary Fig. 4e,f).

Consistent with stabilization of CHK1, overexpression of USP7 markedly reduced the polyubiquitylation level of CHK1 in 293T cells (Fig. 5i). To directly examine the deubiquitylating activity of USP7 towards CHK1, we purified USP7 and ubiquitylated CHK1 and then incubated them in a cell-free system. USP7 purified from 293T cells transfected with USP7 alone decreased CHK1 polyubiquitylation by 25% in vitro, and USP7 and ZEB1 co-purified from 293T cells with co-transfection of USP7 and ZEB1 reduced CHK1 polyubiquitylation by 43% (Fig. 5j). Similar to the knockdown effect of ZEB1 and CHK1, depletion of USP7 also radiosensitized SUM159-P2 cells (Fig. 5k). We conclude from these experiments that CHK1 is a USP7 substrate, and that ZEB1 directly interacts with USP7 and enhances its ability to deubiquitylate and stabilize CHK1, which in turn promotes radioresistance.

Figure 4 CHK1 mediates ZEB1 regulation of radiosensitivity. (a) Immunoblotting of p-CHK1, CHK1, p-CHK2, CHK2 and GAPDH in SUM159-P2 cells transduced with ZEB1 shRNA, at the indicated time points after 6 Gy ionizing radiation (IR). (b) Immunoblotting of ZEB1, CHK1, γH2AX, H2AX and GAPDH in MCF7 cells transduced with ZEB1, at the indicated time points after 6 Gy ionizing radiation. (c) Immunoblotting of ZEB1, CHK1 and GAPDH in Zeb1–/–, Zeb1+/– and Zeb1+/+ MEFs. (d) Immunoblotting of ZEB1, CHK1, cyclin A, p-H3 (S10) and GAPDH in SUM159-P2 cells transfected with ZEB1 siRNA or the scramble control. Cells were arrested overnight with 0.5 μg/ml nocodazole. Mitotic cells were ‘shaken off’ and then released into normal medium. Samples were collected at the indicated time points and analysed by western blotting. Cell cycle distribution was gauged by cyclin A and p-H3 (S10). (e) Clonogenic survival assays of SUM159-P0 cells transfected with CHK1 siRNA. Inset: immunoblotting of CHK1 and GAPDH. n = 3 wells per group. (f) Immunoblotting of CHK1 and GAPDH in ZEB1 shRNA-transduced SUM159-P2 cells with or without ectopic expression of CHK1. (g) Clonogenic survival assays of ZEB1 shRNA-transduced SUM159-P2 cells with or without ectopic expression of CHK1. n = 3 wells per group. Significance of ZEB1 siRNA + Mock versus ZEB1 shRNA + CHK1 is shown. (h) Immunoblotting of ZEB1, CHK1, CHK2, γH2AX, H2AX, p-CHK2, CHK1 and GAPDH in ZEB1 shRNA-transduced SUM159-P0 cells transfected with ZEB1 alone or in combination with CHK1 siRNA. (i) Clonogenic survival assays of SUM159-P0 cells transfected with ZEB1 alone or in combination with CHK1 siRNA. Significance of Scramble + ZEB1 versus CHK1 siRNA + ZEB1 is shown. n = 3 wells per group. Data in e, g and i are the mean of biological replicates from a representative experiment, and error bars indicate s.e.m. Statistical significance was determined by a two-tailed, unpaired Student’s t-test. The experiments were repeated 3 times. For source data, see Supplementary Table 3. Uncropped images of blots are shown in Supplementary Fig. 7.

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Interestingly, ectopic expression of ZEB1 markedly enhanced the interaction of USP7 with CHK1, but not its association with HLTF, p53 or Claspin (Fig. 6a and Supplementary Fig. 4g). Conversely, knockdown of ZEB1 markedly decreased the interaction between USP7 (either overexpressed or endogenous) and CHK1, but not HLTF and p53 (Fig. 6b,c). Therefore, ZEB1 specifically promotes the interaction between USP7 and CHK1.

To further understand why ZEB1 regulates the stability of CHK1 but not the stability of other USP7 substrates (Fig. 5g and Supplementary Fig. 4f), we examined the effect of ZEB1 on the interaction between USP7 and its various substrates. As expected, CHK1, HLTF, p53 and Claspin could be detected in USP7 immunoprecipitates (Fig. 6a and Supplementary Fig. 4g). Interestingly, ectopic expression of ZEB1 markedly enhanced the interaction of USP7 with CHK1, but not its association with HLTF, p53 or Claspin (Fig. 6a and Supplementary Fig. 4g). Conversely, knockdown of ZEB1 markedly decreased the interaction between USP7 (either overexpressed or endogenous) and CHK1, but not HLTF and p53 (Fig. 6b,c). Therefore, ZEB1 specifically promotes the interaction between USP7 and CHK1.
ZEB1 is phosphorylated and stabilized by ATM

We sought to determine the mechanism by which the ZEB1 protein is upregulated in radioresistant cells derived from irradiation. A central component in the DNA repair pathway is ATM (ref. 45); on exposure to ionizing radiation, ATM kinase is rapidly activated, leading to phosphorylation of a number of key players in DDR, cell cycle arrest and apoptosis, such as γH2AX (ref. 8), CHK2 (ref. 46), BRCA1 (ref. 47) and p53 (refs 48,49).

We investigated whether ZEB1 is regulated by ATM. Co-immunoprecipitation revealed physical interaction of ZEB1 with ATM (Fig. 7a), whereas ATR showed no association with ZEB1 (Supplementary Fig. 5a). Moreover, depletion of ATM in SUM159-P2 cells significantly downregulated ZEB1 and CHK1 proteins (Fig. 7b); in contrast, neither knockdown of ATR or treatment with the ATR inhibitor ETP-46464 (ref. 50) affected ZEB1 protein levels (Supplementary Fig. 5b,c). ATM substrates have a common S/T-Q motif. Analysis of the ZEB1 protein sequence revealed one evolutionarily conserved S/T-Q motif encompassing Ser 585. An inhibitor of ATM phosphorylation that ATM-dependent phosphorylation of ZEB1 is important for the regulation of radiation response.

Substitution of either alanine or aspartic acid for Ser 585 (S585A) resulted in a 70–80% decrease in S/T-Q phosphorylation of ZEB1 in irradiated 293T cells (Fig. 7f,g), suggesting that this serine residue accounts for most ZEB1 S/T-Q phosphorylation in cells with activated ATM. To determine whether ZEB1 is a direct substrate of ATM, we purified ZEB1 and ATM and then performed in vitro kinase assays. As a positive control, the known ATM substrate p53 was phosphorylated by wild-type ATM, but not the kinase-dead mutant (S585D) (Fig. 7h). Notably, ATM exhibited robust kinase activity towards wild-type ZEB1, whereas the phosphorylation of the S585A mutant was reduced by 60% (Fig. 7h), which suggested that ATM can directly phosphorylate ZEB1 at Ser 585, but other phosphorylation sites may also exist.

To determine whether ATM can stabilize ZEB1 through phosphorylating it at Ser 585, we compared wild-type ZEB1 with the phosphodeficient (S585A) and phosphomimetic (S585D) mutants. Mutation at Ser 585 did not alter the physical association between ZEB1 and USP7 (Supplementary Fig. 6a) but did affect ZEB1 protein stability: in the absence of ionizing radiation, the stability of wild-type ZEB1 was much higher than that of the S585A mutant but much lower than that of the S585D mutant (Fig. 7i and Supplementary Fig. 6b); in the presence of ionizing radiation, the stability of wild-type ZEB1 was markedly increased to a level as high as that of the S585D mutant, whereas the S585A mutant was much less stable (Fig. 7i and Supplementary Fig. 6c). Therefore, ATM-dependent phosphorylation of ZEB1 at Ser 585 is crucial for ionizing-radiation-induced stabilization of ZEB1 but not the interaction between ZEB1 and USP7. This reveals the underlying mechanism by which ZEB1 protein is upregulated in radioresistant breast cancer cells with hyperactivation of ATM. Finally, in SUM159-P0 cells, the S585A mutant was less able to promote radioresistance than wild-type ZEB1 or the S585D mutant (Fig. 7j), suggesting that ATM-dependent phosphorylation of ZEB1 is important for the regulation of radiation response.

ZEB1 correlates with CHK1 protein levels and poor clinical outcome in human breast cancer

To validate the association between CHK1 and ZEB1 in breast cancer patients, we performed immunohistochemical staining of these two proteins (Fig. 8a) on the breast cancer progression tissue microarrays...
Figure 7 ATM phosphorylates and stabilizes ZEB1. (a) 293T cells were transected with SFB–ZEB1 and treated with ionizing radiation, followed by pulldown with streptavidin–Sepharose beads (s–S) and immunoblotting with antibodies against ATM and FLAG. (b) SUM159-P2 cells were transduced with ATM shRNA and treated with ionizing radiation (IR). Lysates were immunoblotted with antibodies against p-ATM, ATM, ZEB1, CHK1 and GAPDH. (c) SUM159-P2 cells with or without Ku55933 pretreatment (10 µM, 1 h) were treated with ionizing radiation (6 Gy) and CHX (50 µg ml⁻¹), collected at different time points, immunoprecipitated with the ZEB1 antibody and immunoblotted with antibodies against p-S/TQ and ZEB1. (d) 293T cells were transfected with SFB–ZEB1 and treated with ionizing radiation, followed by pulldown with s–S beads and immunoblotting with antibodies against p-S/TQ and ZEB1. (e) Endogenous ZEB1 was immunoprecipitated from SUM159-P0 and SUM159-P2 cells and immunoblotted with antibodies against p-S/TQ and ZEB1. (f) Consensus ATM phosphorylation site on human ZEB1 (Ser 585) and alignment with the conserved site on mouse, rat and Xenopus ZEB1. (g) 293T cells were transfected with wild-type, or the S585A or S585D mutants of SFB–ZEB1 and treated with ionizing radiation, followed by pulldown with s–S beads and immunoblotting with antibodies against p-S/TQ and ZEB1. (h) Immunopurified wild-type ZEB1 or the S585A mutant were incubated with immunopurified wild-type ATM or the kinase-dead (KD) mutant in kinase buffer containing 32P-ATP. After reaction, proteins were resolved by SDS–PAGE and subjected to autoradiography and immunoblotting with antibodies against ZEB1 and p-ATM. Purified GST–p53 was used as a positive control for ATM kinase activity. (i) HeLa cells were co-transfected with SFB–GFP and wild-type, or the S585A or S585D mutants of SFB–ZEB1, treated with CHX with or without IR, collected at different time points and immunoblotted with antibodies against FLAG. SFB–GFP serves as the control for transfection. (j) Clonogenic survival assays of SUM159-P0 cells transfected with wild-type ZEB1 or the mutants. Significance of WT-ZEB1 versus S585A is shown. n = 3 wells per group. Data in j are the mean of biological replicates from a representative experiment, and error bars indicate s.e.m. Statistical significance was determined by a two-tailed, unpaired Student’s t-test. The experiments were repeated 3 times. For source data, see Supplementary Table 3. Uncropped images of blots are shown in Supplementary Fig. 7.

Tumour cells with therapy resistance including radioresistance are likely to be a source of tumour recurrence and metastatic relapse. To determine the correlation of ZEB1 expression with clinical outcome, we analysed a cohort of human breast cancer patients in which transcriptomic profiling was obtained from 286 tumour samples; 87% of these patients received radiotherapy. A highly significant positive correlation (R = 0.43, P < 1 × 10⁻⁶) between CHK1 and ZEB1 was observed in these breast carcinomas, in which 69% (89 of 129) of the tumours with high ZEB1 expression exhibited high CHK1 expression, and 77% (47 of 61) of the tumours with low ZEB1 expression showed low CHK1 expression (Fig. 8b).

Tumour cells with therapy resistance including radioresistance are likely to be a source of tumour recurrence and metastatic relapse.
**DISCUSSION**

Radiation therapy plays an important role in breast cancer management, and one of the main barriers in curing breast cancer is the intrinsic and therapy-induced radioresistant behaviour of tumour cells. Combining chemotherapy with radiation improves outcomes in many cases, but this strategy also increases toxicity. To overcome this obstacle, it is important to identify the critical determinants of radioresistance and to develop safe and effective tumour radiosensitizers.

Recently, a growing body of evidence implicated EMT and cancer stem cells in the acquisition of radioresistance and drug resistance. Here we identified ZEB1 as an ATM substrate and the mechanism underlying the association between EMT and radioresistance (Fig. 8d): in response to ionizing radiation, ATM kinase is activated, which phosphorylates and stabilizes ZEB1; ZEB1 in turn interacts with and promotes the activity of USP7, which deubiquitylates and stabilizes CHK1.

Cul1- and Cul4-containing E3 ubiquitin ligase complexes target CHK1 for polyubiquitylation and degradation during periods of replicative and genotoxic stress. However, whether this ubiquitylation is reversible and can be antagonized by deubiquitylases remains elusive. In this study, we identified CHK1 as a substrate of a ZEB1-associated deubiquitylating enzyme, USP7. How exactly ZEB1 specifically promotes the interaction of USP7 with CHK1 but not with other USP7 substrates warrants future investigation.

ATM kinase is constitutively activated in radioresistant breast cancer cells (Fig. 7b,e), which could explain upregulation of ZEB1 protein in these cells. It should be noted that checkpoint activation and DNA repair normally occur within minutes or hours after DNA damage, whereas the half-life of ZEB1 protein is approximately 24 h (Fig. 7i and Supplementary Fig. 6b,c). Therefore, ATM-mediated stabilization of ZEB1 may not play a major role in the acute response to ionizing radiation, but instead is important for the enhanced DNA repair ability of radioresistant tumour cells with hyperactivated ATM.

Overexpression of ZEB1 has been observed in human breast tumours and other cancer types. Our findings raise the
METHODS

Methods and any associated references are available in the online version of the paper.

AUTHOR CONTRIBUTIONS

P.Z. and L.M. conceived and designed the project. P.Z. performed and analysed the manuscript with input from all other authors. Y.H. contributed to discussion and revision of the manuscript. P.Z. and L.M. wrote the original draft. J.Y. and J.C. provided DR-GFP-expressing U2OS cells and performed tandem-studies. B.G.D. and W.A.W. established the radioresistant subline. Y.Y. and H.L. performed computational data analysis. J.Z. and S.W. performed genomics studies. B.I. and C.T. provided human patient samples. L.W. and K.K.A. performed tumour radiosensitivity studies. B.I. and C.T. supported the NIH grants R00CA135572, R01CA166651 and R01CA181029 (to L.M.) and a CPRIT Scholar Award R1004 (to L.M.). L.M. is an R. Lee Clark Fellow of The University of Texas MD Anderson Cancer Center. B.G.D. and W.A.W. are supported by a Komen Foundation Grant KG101478. Y.H. is supported in part by NIH U54CA151668. We wish to dedicate this work to the memory of K. Khan Ang.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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M E T H O D S

Cell culture. Mouse embryonic fibroblasts were isolated from Zeb1-deficient embryos, genotyped and cultured as previously described. The 293T, MCF7 and HeLa cell lines were from ATCC and cultured under conditions specified by the manufacturer. The SUM159 cell line was from S. Ethier (Medical Oncology of South Carolina, USA) and cultured as described at http://www.asterand.com/Asterand/human_tissues/159PTh.htm. The HMLE cell line was from R. A. Weinberg’s (Whitehead Institute for Biomedical Research, USA) laboratory stock and cultured in complete Mammary Epithelial Cell Growth Medium (MEGM from Lonza). The DR-GFP-expressing U2OS cell line was from J. Masis (Memorial Sloan-Kettering Cancer Center, USA) and cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin and streptomycin.

Plasmids and shRNA. The Snail, Twist and ZEB1 expression constructs were from R. A. Weinberg. Wild-type ATM and the kinase-dead mutant constructs were described previously. The following shRNA and ORF clones were from Open Biosystems through MD Anderson’s shRNA and ORFeome Core: human ZEB1 shRNA, V3LHS-356186 (5′-AGATTTACGGTGCTGCTCC-3′); human ATM shRNA, V3LHS-350469 (5′-TCAAGAAGCACCACCTGGTGG-3′); and V3LHS-350471 (5′-AGTCTTACACGATCTGCTGG-3′); human CHK1 ORF, PLOHS-10005537; human USP7 ORF, PLOHS-10066416. The ZEB1 and USP7 ORFs were sublicensed into the pBabe-SFB vector using the Gateway system (Invitrogen). The RNAi-resistant ZEB1 mutant (ZEB1-RE) was generated using a QuikChange Site-Directed Mutagenesis Kit (Stratagene). The vectors used in this study are listed in Supplementary Table 2.

siRNA oligonucleotides. The following siRNA oligonucleotides were purchased from Sigma: CHK1 siRNA, SASI_Hs02_00326305 (5′-GGAGAGAAGGCGAAUAUCAdTdT-3′); Snail siRNA, SASI_Hs01_00039785 (5′-GGACUGUAGCGCUTGUGGAdTdT-3′); Twist siRNA, SASI_MIN01_00045024 (5′-GGUCACUAGGCCAAUGGCUAdTdT-3′). The on-target plus siRNA that targets ZEB1 was purchased from Dharmacon (J-006564-10-005, 5′-CUGAGGGAGAAGGCCGAA-3′). Cells were transfected with 150 nM of the indicated oligonucleotide using the Oligofectamine reagent (Invitrogen). Forty-eight hours after siRNA transfection, cells were used for functional assays, and the remaining cells were collected for western blot analysis.

RNA isolation and real-time PCR with reverse transcription. Total RNA was isolated using the mirVana RNA Isolation Kit (Ambion) and was then reverse transcribed with an iScript cDNA Synthesis Kit (Bio-Rad). The resulting cDNA was used for quantitative PCR using the TaqMan Gene Expression Assays (Applied Biosystems), and data were normalized to an endogenous control GAPDH. Real-time PCR and data collection were performed on a CFX96 instrument (Bio-Rad).

Lentiviral and retroviral transduction. The production of lentivirus and amphotropic retrovirus and infection of target cells were performed as described previously.

Immunoblotting. Western blot analysis was performed with precast gradient gels (Bio-Rad) using standard methods. Briefly, cells were lysed in the RIPA buffer containing protease inhibitors (Roche) and phosphatase inhibitors (Sigma). Proteins were separated by SDS–PAGE and blotted onto a nitrocellulose membrane (Bio-Rad). Membranes were probed with the specific primary antibodies, followed by peroxidase-conjugated secondary antibodies. The bands were visualized by chemiluminescence (Denville Scientific). The following antibodies were used: antibodies against ZEB1 (1:1,000, Bethyl Laboratories, A301-922A), CHK1 (1:1,000, Santa Cruz Biotechnology, sc-8048) and ZEB1 (1:100, Bethyl Laboratories, A301-922A). Cells were transfected with 10 μg of wild-type FLAG–ATM or the kinase-dead mutant and then irradiated. Activated or kinase-dead ATM was immunopurified from the cell extracts with FLAG beads (Sigma, M8823).

Deubiquitylation of CHK1 in vivo and in vitro. For the in vivo deubiquitylation assay, transfected 293T cells were treated with a proteasome inhibitor MG132 (10 μM) for 6 h. The cell extracts were subjected to immunoprecipitation and western blot analysis with the indicated antibodies. For preparation of ubiquitlated CHK1 as the substrate for the in vitro deubiquitylation assay, 293T cells were co-transfected with HA–ubiquitin and SFB–CHK1 and were treated with MG132 for 6 h. Ubiquitlated CHK1 was purified from the cell extracts with streptavidin–Sepharose beads. After extensive washing with NETN buffer, the bound proteins were eluted with biotin. In vitro deubiquitylation reaction was performed as described previously. Briefly, ubiquitlated CHK1 protein was incubated with purified USP7 in deubiquitylation buffer (50 mM Tris·HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol and 5% glycerol) for 2 h at 37 °C. After reaction, CHK1 was immunoprecipitated with FLAG antibody–conjugated beads. The beads were washed with deubiquitylation buffer, and the bound proteins were eluted by boiling in 1× Laemmli buffer and subjected to western blot analysis with the indicated antibodies.

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Three mutually orthogonal diameters of the tumour were measured every other day with a vernier caliper, and the mean value was calculated and used as the tumour diameter. An investigator (L.W.) who measured tumour size was blinded to the group allocation during all animal experiments and outcome assessment. General linear model multivariate analysis was performed to determine statistical significance using the SPSS 14.0 software package.

**Patient study.** The breast cancer progression tissue microarrays were purchased from the NCI Cancer Diagnosis Program. These tissue microarrays consist of three different case sets, including 190 analyzable cases of breast carcinoma. Samples were deparaffinized and rehydrated. Antigen retrieval was done by using 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven. To block endogenous peroxidase activity, the sections were treated with 1% hydrogen peroxide in methanol for 30 min. After 1 h pre-incubation in 10% normal serum to prevent nonspecific staining, the samples were incubated with the antibodies against ZEB1 (1:400, Bethyl Laboratories, A301-922A) and CHK1 (1:150, Santa Cruz Biotechnology, sc-7234) at 4°C overnight. The sections were then incubated with a biotinylated secondary antibody, followed by incubation with avidin–biotin peroxidase complex solution (1:100) for 1 h at room temperature. Colour was developed with the 3- amino-9-ethylcarbazole (AEC) solution. Counterstaining was carried out using Mayer's haematoxylin. All immunostained slides were scanned on the Automated Cellular Image System III (ACIS III) for quantification by digital image analysis. A total score of protein expression was calculated from both the percentage of immunopositive cells and immunostaining intensity. High and low protein expression was defined using the mean score of all samples as a cutoff point. The $\chi^2$ test was used for statistical analysis of the correlation between ZEB1 and CHK1.

**Statistical analysis.** Each experiment was repeated three times or more. Unless otherwise noted, data are presented as mean ± s.e.m., and Student's $t$-test (unpaired, two-tailed) was used to compare two groups for independent samples. The data analysed by $t$-test meet normal distribution; we used an $F$-test to compare variances, and the variances are not significantly different. Therefore, when using an unpaired $t$-test, we assumed equal variance, and no samples were excluded from the analysis. $P < 0.05$ was considered statistically significant.

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Supplementary Figure 1 Induction of EMT by Snail, Twist or ZEB1. (a) Phase contrast images of HMLE cells transduced with Snail, Twist or ZEB1. Scale bar: 50 μm. (b) Images of clonogenic assays of HMLE cells transduced with Snail, Twist or ZEB1. (c) Immunoblotting of ZEB1 and GAPDH in mock-infected HMLE cells or HMLE cells transduced with ZEB1 alone or in combination with transfection of ZEB1 siRNA. C: control (non-irradiated); SF: survival fraction collected 3 weeks after 6-Gy irradiation.
Supplementary Figure 2. ZEB1, SNAI1 and TWIST1 mRNA levels are not substantially increased in SUM159-P2 cells. (a) qPCR of ZEB1, SNAI1 and TWIST1 in SUM159-P0 and SUM159-P2 cells. n = 3 samples per group. (b) Clonogenic survival assays of U2OS cells transfected with ZEB1 siRNA. n = 3 wells per group. Inset: immunoblotting of ZEB1 and GAPDH. Data in a and b are the mean of biological replicates from a representative experiment, and error bars indicate s.e.m. Statistical significance was determined by a two-tailed, unpaired Student’s t-test. The experiments were repeated 3 times. The source data can be found in Supplementary Table 3. Uncropped images of blots are shown in Supplementary Fig. 7.
**Supplementary Figure 3** Effect of ZEB1 on CHK1, radiosensitivity and the G2 checkpoint. (a) Immunoblotting of ZEB1, γH2AX, H2AX and GAPDH in ZEB1 shRNA-transduced SUM159-P2 cells with or without ectopic expression of an RNAi-resistant ZEB1 mutant (ZEB1-RE), at the indicated time points after 6 Gy IR. (b) Clonogenic survival assays of ZEB1 shRNA-transduced SUM159-P2 cells with or without ectopic expression of an RNAi-resistant mutant (ZEB1-RE). *n* = 3 wells per group. (c) Percentage of the G2/M population. SUM159-P2 cells were transduced with ZEB1 shRNA, treated with 6-Gy IR and analyzed by flow cytometry. *n* = 3 wells per group. Data in b and c are the mean of biological replicates from a representative experiment, and error bars indicate s.e.m. Statistical significance was determined by a two-tailed, unpaired Student’s *t*-test. The experiments were repeated 3 times. The source data can be found in Supplementary Table 3. Uncropped images of blots are shown in Supplementary Fig. 7.
**Supplementary Figure 4** ZEB1 specifically regulates the protein stability of the USP7 target CHK1. (a) qPCR of CHK1 in SUM159-P2 cells transduced with ZEB1 shRNA. n = 3 samples per group. (b) Quantification of CHK1 protein levels (normalized to GAPDH) in Fig. 5f. (c) Quantification of CHK1 protein levels (normalized to GAPDH) in Fig. 5g. (d) SUM159-P2 cells were transfected with the scramble control or USP7 siRNA (si-USP7) and then treated with 50 μg/ml cycloheximide (CHX). Cells were harvested at different time points as indicated and then immunoblotted with antibodies to ZEB1, USP7 and GAPDH. (e) Quantification of CHK1 protein levels (normalized to GAPDH) in Fig. 5h. (f) SUM159-P2 cells were treated with 50 μg/ml cycloheximide (CHX), harvested at different time points as indicated and then immunoblotted with antibodies to Claspin, ZEB1 and GAPDH. (g) 293T cells were transfected with SFB-USP7 alone or in combination with ZEB1, followed by pull-down with streptavidin-sepharose beads (s-s beads) and immunoblotting with the antibody to Claspin. Data in a are the mean of biological replicates from a representative experiment, and error bars indicate s.e.m. Statistical significance was determined by a two-tailed, unpaired Student's t-test. The experiments were repeated 3 times. The source data can be found in Supplementary Table 3. Uncropped images of blots are shown in Supplementary Fig. 7.
**Supplementary Figure 5** ATR does not regulate ZEB1. (a) 293T cells were transfected with SFB-ZEB1, followed by pull-down with streptavidin-sepharose beads (s-s beads) and immunoblotting with antibodies to ATR and ATM. (b) SUM159-P2 cells were transfected with ATR siRNA and treated with IR. Lysates were immunoblotted with antibodies to p-ATR, ATR, ZEB1 and GAPDH. (c) SUM159-P2 cells were pretreated with ETP-46464 and treated with IR. Lysates were immunoblotted with antibodies to ZEB1, p-CHK1, CHK1, p-CHK2, CHK2 and GAPDH.
Supplementary Figure 6 ATM-dependent phosphorylation of ZEB1 at S585 is critical for radiation-induced stabilization of ZEB1 but not the interaction between ZEB1 and USP7. (a) 293T cells were transfected with SFB-ZEB1 (wild-type, S585A or S585D), followed by pull-down with streptavidin-sepharose beads (s-s beads) and immunoblotting with the USP7 antibody. (b, c) Quantification of ZEB1 proteins levels (normalized to co-transfected GFP) in Fig. 7i. Uncropped images of blots are shown in Supplementary Fig. 7.
Supplementary Figure 7 Uncropped images of blots.
Supplementary Figure 7 continued
Supplementary Figure 7 continued
Supplementary Figure 7 continued
Supplementary Figure 7 continued
Supplementary Figure 7 continued
Supplementary Figure 7 continued
Fig. 7a

Supplementary Figure 7 continued
Supplementary Figure 7 continued
Supplementary Figure 7 continued
**Supplementary Figure 4d**

- ZEB1
- USP7
- GAPDH

**Supplementary Figure 4g**

- Claspin
- FLAG-USP7
- GAPDH
- ZEB1

**Supplementary Figure 4f**

- Claspin
- ZEB1
- GAPDH

Supplementary Figure 7 continued
Supplementary Figure 5c

Supplementary Figure 5a

Supplementary Figure 6a
**Supplementary Table 1** List of ZEB1-interacting proteins identified by TAP-MS analysis

| Peptide hits | Protein Name |
|--------------|--------------|
| 44           | ZEB1         |
| 39           | C12orf11     |
| 22           | C15orf44     |
| 16           | CTBP2        |
| 15           | XRCC5        |
| 14           | INTS10       |
| 13           | XRCC6        |
| 11           | DDB1         |
| 9            | CTBP1        |
| 8            | SIRT1        |
| 8            | AHCYL1       |
| 8            | PPM1G        |
| 5            | TP53         |
| 5            | CSNK2A1      |
| 5            | AHCYL2       |
| 5            | MYBBP1A      |
| 4            | USP7         |
| 3            | C7orf26      |
| 3            | LRRC59       |
| 3            | FAM98B       |
| 3            | PUF60        |
| 3            | TRIM28       |
| 3            | ZNF516       |
| 2            | RIOK2        |
| 2            | CHFR         |
| 2            | SSRP1        |
| 2            | MTDH         |
| 2            | PARP1        |
| 2            | C22orf28     |
| 2            | SART1        |
| 1            | WIBG         |
| 1            | LTV1         |
| 1            | ZNF655       |
| 1            | CROP         |
| 1            | LUC7L2       |
| 1            | ICT1         |
| 1            | U2AF1        |
| 1            | AFG3L2       |
| 1            | U2AF2        |
| 1            | C10orf125    |
| 1            | WDR12        |
| 1            | LUC7L2       |
| 1            | ERI1         |
| 1            | FAU          |
| 1            | STRBP        |
| 1            | PHF6         |
| 1            | AIMP1        |
**Supplementary Table 2** Vectors used in this study

| Insert name            | Vector name |
|------------------------|-------------|
| Mouse Twist1 ORF       | pBabe-puro  |
| Human SNAI1 ORF        | pBabe-puro  |
| Human ZEB1 ORF         | pFUW        |
| Human CHK1 ORF         | pLOC-blast  |
| Human ZEB1 shRNA       | pGIPZ-puro  |
| Human ATM shRNA        | pGIPZ-puro  |
| Human ATM ORF          | pcDNA3      |
| Human USP7 ORF         | pBabe-SFB   |
Supplementary Table 3 Source data.