AKT and EZH2 inhibitors kill TNBCs by hijacking mechanisms of involution

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Amy E. Schade^{1,2}, Naiara Perurena^{1,2}, Yoona Yang^{3,4}, Carrie L. Rodriguez^{1,2}, Anjana Krishnan^{1,2}, Alycia Gardner^{1,2}, Patrick Loi^{1,2}, Yilin Xu^{5,6,7}, Van T. M. Nguyen⁸, G. M. Mastellone^{1,2}, Natalie F. Pilla^{1,2}, Marina Watanabe^{1,2}, Keiichi Ota⁹, Rachel A. Davis^{1,2}, Kaia Mattioli^{1,2}, Dongxi Xiang^{1,2}, Jason J. Zoeller^{5,10}, Jia-Ren Lin^{5,6,7}, Stefania Morganti^{2,9}, Ana C. Garrido-Castro^{2,9}, Sara M. Tolaney^{2,9}, Zhe Li^{1,2}, David A. Barbie⁹, Peter K. Sorger^{5,6,11}, Kristian Helin⁸, Sandro Santagata^{5,6,7,11}, Simon R. V. Knott^{3,4} & Karen Cichowski^{1,2,5}

Triple-negative breast cancer (TNBC) is the most aggressive breast cancer subtype and has the highest rate of recurrence¹. The predominant standard of care for advanced TNBC is systemic chemotherapy with or without immunotherapy; however, responses are typically short lived^{1,2}. Thus, there is an urgent need to develop more effective treatments. Components of the PI3K pathway represent plausible therapeutic targets; more than 70% of TNBCs have alterations in PIK3CA, AKT1 or PTEN³⁻⁶. However, in contrast to hormone-receptor-positive tumours, it is still unclear whether or how triple-negative disease will respond to PI3K pathway inhibitors⁷. Here we describe a promising AKT-inhibitor-based therapeutic combination for TNBC. Specifically, we show that AKT inhibitors synergize with agents that suppress the histone methyltransferase EZH2 and promote robust tumour regression in multiple TNBC models in vivo. AKT and EZH2 inhibitors exert these effects by first cooperatively driving basal-like TNBC cells into a more differentiated, luminal-like state, which cannot be effectively induced by either agent alone. Once TNBCs are differentiated, these agents kill them by hijacking signals that normally drive mammary gland involution. Using a machine learning approach, we developed a classifier that can be used to predict sensitivity. Together, these findings identify a promising therapeutic strategy for this highly aggressive tumour type and illustrate how deregulated epigenetic enzymes can insulate tumours from oncogenic vulnerabilities. These studies also reveal how developmental tissue-specific cell death pathways may be co-opted for therapeutic benefit.

The PI3K pathway is frequently hyperactivated in breast cancers³⁻⁵. In hormone receptor-expressing (HR⁺) luminal tumours, the most common mutations affect *PIK3CA*, which encodes the catalytic p110 α subunit of PI3K^{8,9}. Accordingly, a PI3K α -selective inhibitor, combined with an oestrogen receptor (ER) antagonist, is approved for advanced HR⁺ disease^{10,11}. However, *PTEN* mutations are more prevalent in basal-like TNBCs, which can function through PI3K p110 $\beta^{3,10}$. Thus, agents that target downstream components, such as AKT, may be required to suppress PI3K signalling in these tumours. Nevertheless, an AKT inhibitor plus paclitaxel did not improve outcomes in a phase III TNBC trial⁷, although studies with other agents are pending¹². As such, it is still unclear whether or how PI3K pathway inhibitors will be useful in this aggressive breast cancer subtype.

Given the efficacy of PI3K inhibitors in luminal tumours, we investigated whether TNBCs could be sensitized to AKT inhibitors by modulating cell state. EZH2 is the catalytic component of PRC2, which promotes gene silencing by methylating histone H3 on lysine 27 (H3K27me3)¹³. EZH2 has a broad role in stem cell maintenance, and its overexpression promotes the development of many cancers, including TNBC¹³⁻¹⁶. EZH2 is also required to maintain luminal progenitors and restrict luminal cell differentiation in mice^{17,18}, whereas its targeted overexpression drives intraductal hyperplasia¹⁹. Although EZH2 inhibitors do not affect the growth of established breast cancers, EZH2 suppression impairs TNBC metastasis²⁰. We therefore initially hypothesized that EZH2 inhibitors might sensitize TNBCs to AKT inhibitors by converting these poorly differentiated tumours to a more luminal-like state. Fortunately, several

¹Division of Genetics, Brigham and Women's Hospital, Boston, MA, USA. ²Department of Medicine, Harvard Medical School, Boston, MA, USA. ³Cedars-Sinai Samuel Oschin Comprehensive Cancer Institute, Los Angeles, CA, USA. ⁴Department of Biomedical Sciences, Cedars-Sinai Medical Center, Los Angeles, CA, USA. ⁵Ludwig Center at Harvard, Harvard Medical School, Boston, MA, USA. ⁶Laboratory of Systems Pharmacology, Harvard Medical School, Boston, MA, USA. ⁷Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA. ⁸Division of Cancer Biology, Institute of Cancer Research, London, UK. ⁹Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA. ¹⁰Department of Cell Biology, Harvard Medical School, Boston, MA, USA. ¹⁰Department of Systems Biology, Harvard Medical School, Boston, MA, USA. ¹⁰Department of Cell Biology, Harvard Medical School, Boston, MA, USA. ¹⁰Department of Cell Biology, Harvard Medical School, Boston, MA, USA. ¹⁰Department of Cell Biology, Harvard Medical School, Boston, MA, USA. ¹⁰Department of Cell Biology, Harvard Medical School, Boston, MA, USA. ¹⁰Department of Cell Biology, Harvard Medical School, Boston, MA, USA. ¹⁰Department of Cell Biology, Harvard Medical School, Boston, MA, USA. ¹⁰Department of Cell Biology, Harvard Medical School, Boston, MA, USA. ¹⁰Department of Cell Biology, Harvard Medical School, Boston, MA, USA. ¹⁰Department of Cell Biology, Harvard Medical School, Boston, MA, USA. ¹⁰Department of Cell Biology, Harvard Medical School, Boston, MA, USA. ¹⁰Department of Cell Biology, Harvard Medical School, Boston, MA, USA. ¹⁰Department of Cell Biology, Harvard Medical School, Boston, MA, USA. ¹⁰Department of Cell Biology, Harvard Medical School, Boston, MA, USA. ¹⁰Department of Cell Biology, Harvard Medical School, Boston, MA, USA. ¹⁰Department OSA, ¹⁰Department Cell Biology, Harvard Medical School, Boston, MA, USA, ¹⁰Department Cell Biology, Harvard Medical School, Boston, MA, USA



Fig. 1| AKT and EZH2 inhibitors potently synergize, kill TNBCs and promote substantial tumour regression in vivo. a, EZH2 mRNA levels in TNBC tumours (n = 116) and matched normal breast tissue (n = 112). The dashed line indicates 2 s.d. above the mean of normal. The box limits show the 25th-75th percentiles, whiskers show 10th-90th percentiles, and centre line shows median. b, Multiplexed CyCIF analysis of TNBC tumour and normal breast epithelium stained for E-cadherin (cyan) and EZH2 (magenta) and with Hoechst. Scale bars, 50 µm. c, The relative change in cell number after 4 days of treatment with AKTi (ipatasertib) and/or EZH2i (tazemetostat) (day 4 (D4) versus day 0 (D0)). P values were calculated using unpaired one-tailed heteroscedastic Student's t-tests. n = 3. Data are mean \pm s.d. of biologically independent samples. P values from left to right are as follows: 6.27 × 10⁻⁴, 3.02 × 10⁻², 1.30 × 10⁻⁴, 4.24 × 10⁻³, $2.57 \times 10^{-4}, 1.10 \times 10^{-5}, 2.12 \times 10^{-3}, 2.98 \times 10^{-3}, 1.88 \times 10^{-2}, 7.66 \times 10^{-3}, 5.76 \times 10^{-$ 2.28 × 10⁻⁴. FC, fold change. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. **d**, Live-cell imaging of dying SUM149PT cells in response to the indicated treatments. P values were determined using two-way analysis of variance (ANOVA). n = 8 technical

EZH2 inhibitors have been developed and one, tazemetostat, is FDA approved for other indications^{21,22}.

AKT and EZH2 inhibitors kill TNBCs

EZH2 is overexpressed in 82% of TNBCs and is readily detected in tumour cell nuclei (Fig. 1a,b and Extended Data Fig. 1a). A panel of TNBC cell lines were pretreated with tazemetostat for 5 days, followed by the addition of the AKT inhibitor ipatasertib (Extended Data Fig. 1b). 60% of cell lines were highly sensitive to this combination, resulting in a substantial depletion of cells within 4 days (Fig. 1c and Extended Data Fig. 1c,d). Live-cell imaging further demonstrated that AKT and EZH2 inhibitors do not effectively kill TNBCs as single agents but do so when combined, in a dose-dependent manner (Fig. 1d and Extended Data Fig. 1e). Notably, AKT and EZH2 inhibitors were highly synergistic in all sensitive cell lines, with highest single agent (HSA) scores of \geq 10

replicates. **e**, Synergy plot using an HSA model for cells treated with AKTi and EZH2i. **f**-**i**, Waterfall plots of MDA-MB-468 xenografts (n = 10-13 tumours; **f**), SUM149PT xenografts (n = 8-14 tumours; **g**), GEMM allografts (n = 8-10 tumours; **h**) or PDX HC1-004 (n = 8-12 tumours; **i**) after 28 days of treatment with vehicle (veh.), AKTi (ipatasertib) and/or EZH2i (tazemetostat) (17 days for GEMM allograft). All tumours were orthotopic. *P* values were calculated using two-tailed Mann–Whitney *U*-tests. **j**, The relative tumour volume of PDX HCI-004 (n = 8-12 tumours) over the treatment course of 52 days. Data are mean \pm s.e.m. of biologically independent samples. **k**, Multiplexed CyCIF analysis of PDX-004 tumours (entire tumour section) treated with vehicle or EZH2/AKTi after 1 or 2 days and stained with antibodies against pan-cytokeratin (pan-CK, cellular, blue), Ki-67 (proliferation, pink) and cPARP (apoptosis, white). Scale bars, 500 µm. **I**, Waterfall plot of PDX HCI-025 (n = 8-10 tumours each) after 28 days of treatment. Sub-labeled bars correspond with histology in Extended Data Fig. 4.

(Fig. 1e and Extended Data Fig. 1f). Similar effects were observed when ipatasertib was replaced with a different AKT inhibitor (Extended Data Fig. 2a) or when tazemetostat was substituted with an EED inhibitor, which suppresses another obligate PRC2 component (Extended Data Fig. 2b). These agents did not exert cytotoxic effects in non-transformed epithelial cells or in other primary or unrelated cell lines (Extended Data Fig. 2c,d). Responses to combined agents were durable, in contrast to the effects of ipatasertib alone, which slowed cell proliferation but permitted regrowth (Extended Data Fig. 2e). Sequential dosing of tazemetostat followed by ipatasertib exerted a similar cytotoxic response, but the effects were less robust when a 4 day gap between drugs was included, suggesting that, although concomitant dosing may not be required, maximal responses require a sustained loss of H3K27me3 (Extended Data Fig. 2f).

EZH2 was overexpressed in all cell lines, and the relative expression levels did not correlate with sensitivity (Extended Data Fig. 3a); however, PTEN loss was enriched in sensitive cells (Fisher's exact test, P = 0.045; Extended Data Fig. 3b). Specifically, PTEN was absent in all of the sensitive lines except for one that had a *PIK3CA* mutation (Extended Data Fig. 3b), and was retained in all but one of the resistant lines, although a second line had a *PIK3CA* mutation (Extended Data Fig. 3b). Thus, although aberrant PI3K pathway activation, often conferred by PTEN loss in TNBCs, probably contributes to sensitivity to this combination, it is not the only determinant.

Xenograft, GEMM-derived allograft and patient-derived xenograft (PDX) models were used to evaluate efficacy in vivo. Mice with established orthotopic tumours (>100 mm³) were pretreated with EZH2 inhibitor (EZH2i) for 1 week, before addition of the AKT inhibitor (AKTi). No or modest cytostatic responses were observed with single agents (Fig. 1f-l and Extended Data Fig. 3c), but, when combined, EZH2i + AKTi (EZH2/AKTi) triggered potent tumour regression in all five models. MDA-MB-468 and SUM149PT xenografts regressed 42-73% over 28 days (Fig. 1f,g), and survival increased from 46 to 116 days, despite treatment cessation (Extended Data Fig. 3d). Notably, this drug combination was well tolerated in vivo (Extended Data Fig. 3e). EZH2/AKT inhibitors similarly induced regression of p53-mutant GEMM-derived TNBC organoid allografts (Fig. 1h), in which PI3K-AKT is hyperactivated by spontaneous AKT3 and MET copy-number gains^{23,24} and EZH2 is amplified and overexpressed (Extended Data Fig. 3g,h). Importantly, EZH2/AKT inhibitors were effective in patient-derived xenograft models. HCI-004 tumours²⁵, which exhibit PTEN copy loss and overexpress EZH2, regressed 60-88%, with one complete response (Fig. 1i). Tumour regression was durable throughout the entire study (7.5 weeks) (Fig. 1j). Multiplexed cyclic immunofluorescence (CycIF) imaging of PDX tumours treated in vivo demonstrated a potent loss of Ki-67 (pink) and concomitant induction of cleaved PARP (white) within 1 day of treatment (Fig. 1k). HCI-025 tumours²⁵, which have a *PIK3CA* mutation, *PTEN* copy loss and *EZH2* overexpression, also regressed 28-57% (Fig. 1l). Although frank regression was more variable, histological analysis of tumours that appeared to arrest rather than shrink revealed a substantial loss of tumour cells internally with extensive necrosis, fibrosis and calcification (Extended Data Fig. 4). Residual viable tumour cells at the end point comprised only 10-40% of the area and were largely devoid of Ki-67 (Extended Data Fig. 4). Thus, the remaining tissue consisted mostly of extracellular matrix and necrotic deposits, with a small number of arrested cells, consistent with the durable response of HCI-004 tumours (Fig. 1i). Potent tumour regression, confirmed here in multiple PDX, xenograft and GEMM models, is rarely observed in TNBC, underscoring the potential therapeutic use of this combination.

Differentiation is required for the response

RNA sequencing (RNA-seq) analysis was performed on cells treated with EZH2 and/or AKT inhibitors after 24 h, before the onset of substantial cell death. Notably, the combination triggered a potent suppression of genes associated with basal and mammary stem cells (such as *KRT5, KRT14* and *VIM*), and the concomitant upregulation of luminal markers (such as *GATA3* and *ELF3*)²⁶ (Extended Data Fig. 5a). While EZH2 inhibitors have been reported to modestly increase the expression of some luminal markers in a TNBC model²⁰, single-sample gene set enrichment analysis (ssGSEA) revealed that EZH2 and AKT inhibitors were both required to maximally induce luminal signatures and suppress basal and mammary stem cell signatures in TNBCs²⁶ (Fig. 2a and Extended Data Fig. 5b). Transcriptional heat maps of the most highly upregulated and downregulated genes illustrate the cooperative nature of this response (Fig. 2b and Extended Data Fig. 5c).

Transcriptional profiles have been used to classify TNBC tumours into basal-like 1 (BL1), basal-like 2 (BL2), mesenchymal and luminal-AR subtypes (termed TNBCtypes)^{27,28}. In SUM149PT cells EZH2/AKTi cooperatively suppressed the BL2 signature and induced the TNBCtype luminal-AR tumour signature (Fig. 2c). A similar suppression of basal-like

TNBCtype signatures was observed in all five sensitive lines evaluated (Fig. 2c and Extended Data Fig. 5d). Notably, ER was not upregulated by EZH2/AKTi, but AR expression did increase slightly (Extended Data Fig. 5e, f). Regardless, these findings suggest that EZH2 and AKT inhibitors cooperatively drive basal TNBCs to a more luminal-like cell state.

To determine whether this transformation was occurring in vivo, further CyCIF imaging was conducted on HCI-004 PDX tumours (Fig. 2d). Vehicle-treated tumours predominantly expressed the basal cytokeratin (CK14, cyan) and minimally expressed the luminal cytokeratin (CK8, magenta) (Fig. 2d). However, EZH2/AKTi induced a marked shift in cell state, with a concomitant loss of basal and gain of luminal markers (that is, CK14 to CK8) (Fig. 2d (top)). Moreover, although cleaved PARP was minimally detected in vehicle-treated tumours, it was highly expressed in CK8 expressing cells after 1-2 days of treatment, indicating that cells with luminal features were rapidly undergoing apoptosis (Fig. 2d (bottom)). A ring of CK14-positive cells remained on the exterior of the tumour for the first few days; however, after 30 days it appeared as if the hollowed tumour core collapsed, leaving CK14-positive cells and a smaller fraction of CK8-positive cells (Fig. 2d). At this timepoint, tumour cells were no longer dying, illustrated by the absence of PARP cleavage (Fig. 2d (bottom)), but residual tumour remnants exhibited minimal proliferation compared to vehicle-treated tumours (Fig. 2e), similar to what was observed in PDX HCI-025 (Extended Data Fig. 4). These results demonstrate that EZH2 and AKT inhibitors drive TNBCs into a luminal-like state in vivo, which results in rapid cell death and durable tumour regression.

Consistent with the induction of a luminal-like cell state, AKTi and EZH2i were both required to induce maximal expression of GATA3, a master developmental transcription factor that controls luminal differentiation^{29,30}, which was confirmed by mRNA and protein expression (Fig. 2f,g and Extended Data Fig. 5g). Moreover, GATA3 ablation using siRNAs, short hairpin RNAs (shRNAs) or sgRNAs prevented the cytotoxic response and the synergy observed between AKTi and EZH2i in multiple cell lines in vitro (Fig. 2h and Extended Data Fig. 5h-k) and in vivo (Fig. 2i). Notably, in the absence of GATA3, EZH2/AKTi still exerted cytostatic effects (Fig. 2h,i), consistent with known individual effects of AKTi and EZH2i on the expression of various cell cycle genes^{31,32}. While the individual contributions of EZH2i and AKTi were not investigated exhaustively, upregulation of p27 and concomitant suppression of the RB-E2F pathway was consistently observed in response to ipatasertib, which was unaffected by GATA3 ablation (Extended Data Fig. 51-n). These findings suggest that GATA3-mediated differentiation is essential for tumour regression, but AKTi and EZH2i can exert effects on other targets, which may impede the proliferation of the remaining cells.

Predicting response by machine learning

This luminal-like differentiation program was defective in resistant cells. For example, studies using assay for transposase-accessible chromatin with sequencing (ATAC-seq) revealed that GATA3-binding sites became accessible in response to EZH2/AKTi only in sensitive cells (Fig. 3a) and that GATA3 expression was not induced by the combination in resistant cells (Fig. 3b). Principal component analysis of ATAC-seq data from untreated cells further showed that sensitive and resistant cells could be readily differentiated by baseline epigenetic differences (Fig. 3c). Notably, in sensitive cells, the top enriched genes associated with open chromatin were luminal and basal genes, whereas mesenchymal gene loci were identified as the most differentially accessible in resistant cells (Extended Data Fig. 6a). This was accompanied by the enrichment of either basal or mesenchymal genes in sensitive versus resistant cells, respectively (Extended Data Fig. 6b,c). These findings suggest that sensitive cells may be poised to undergo a luminal-like state change, whereas resistant cells appear to be inherently less differentiated or more mesenchymal, with a chromatin state that may restrict this differentiation program.



Fig. 2 | Combined EZH2/AKT inhibitors are required for maximal differentiation and the therapeutic response. a, ssGSEAz scores of the LIM_MAMMARY_LUMINAL_MATURE_UP and LIM_MAMMARY_STEM_CELL_UP gene signatures in SUM149PT cells. The box limits show the range of data and the centre lines show the mean. b, Transcriptional heat map depicting expression of significantly differentially expressed genes in gene signatures from a. MaSC, mammary stem cells. c, The correlation coefficient of the SUM149PT transcriptional profile with the luminal-AR and BL2 (basal-like 2) TNBCtypes. d,e, Multiplexed immunofluorescence analysis of HCI-004 PDX orthotopic tumours treated with vehicle or EZH2/AKTi and collected after 1, 2 or 30 days of treatment stained with CK14 (basal, cyan), CK8 (luminal, magenta) and cPARP (apoptosis, yellow) antibodies (d) or at day 30 after treatment and stained with pan-CK (cellular; white) and Ki-67 (proliferation; magenta) antibodies (e). The images represent entire tumour sections and are the same

Despite the baseline differences, TNBCtype or broad mesenchymal signatures were not sufficient to predict sensitivity versus resistance to EZH2/AKTi. Thus, a machine learning approach was performed using CCLE RNA-seq data, which showed little variance from transcriptional data generated in this study (Fig. 3d and Extended Data Fig. 6d). Specifically, we used a leave-one-out cross-validation strategy using transcriptional and sensitivity data from the 12 cell lines in Fig. 1c together with 5 additional cell lines (Extended Data Fig. 6e). For each iteration, either the most variable or the most differentially expressed genes between sensitive and insensitive lines were used in the training set, as specified in Fig. 3d. Random forest (RF) and support vector machine (SVM) algorithms were then evaluated for their ability to predict the sensitivity status of the left-out cell lines. We found that models that were trained using the RF algorithm on genes differentially expressed with an absolute log-transformed fold change of >5 could most accurately predict sensitivity (Extended Data Fig. 6f). When this model

as in Fig. 1k with different channels. **f**, GATA3 expression was measured using qPCR with reverse transcription (RT-qPCR) in cell lines after AKTi (ipatasertib) and/or EZH2i (tazemetostat) treatment. n = 3. **g**, Immunoblot depicting GATA3 expression after treatment with EZH2i and/or AKTi in two sensitive cell lines. The experiment was repeated at least three times. **h**, The relative change in cell number of SUM149PT transfected with siControl (siCtrl) or siGATA3 (left) or transduced with sgControl (sgCtrl) or sgGATA3 (right) and then treated with vehicle, AKTi (ipatasertib) and/or EZH2i (tazemetostat). Data are mean \pm s.d. of biologically independent samples. n = 3. *P* values were calculated using unpaired one-tailed heteroscedastic Student's *t*-tests. **i**, The relative tumour volume of MDA-MB-468 orthotopic xenografts transduced with sgControl or sgGATA3 and treated with vehicle or EZH2/AKTi (tazemetostat + ipatasertib). Data are mean \pm s.e.m. n = 6-9 tumours. Scale bars 500 µm (**d**) and 100 µm (**e**).

was applied to transcriptional data from TCGA Firehose TNBC tumour samples, 55% of tumours were predicted to be sensitive to EZH2/AKTi, (Fig. 3d), similar to the empirical observation that 60% of TNBC cell lines were indeed sensitive to these agents. Notably, while *PTEN* alterations were enriched in sensitive cell lines, closer analysis of TCGA tumours suggests that around 93% of TNBCs have at least one alteration in a PI3K pathway component (*PTEN*, *PI3KCA*, *AKT3* and *INPP4B*; Extended Data Fig. 6g). We therefore postulate that the signature generated here will probably be a better predictor of sensitivity, which can be evaluated in future clinical trials.

Cooperative GATA3 induction through FOXO1

As GATA3 was essential for the therapeutic response, we sought to elucidate the precise contribution of EZH2 and AKT inhibition in its induction. GATA3 is known to be a direct target of PRC2³³. Nevertheless,



Fig. 3 | **Identifying a sensitive TNBC cell state using a machine learning approach. a**, ATAC-seq motif footprinting score for GATA3-binding motifs in cells treated with EZH2i and/or AKTi. **b**, Immunoblot of GATA3 expression after EZH2/AKTi (tazemetostat + ipatasertib) treatment in sensitive and resistant cell lines. The experiment was repeated at least three times. **c**, Principal component analysis of the chromatin landscape of TNBC cell lines as

assessed by ATAC-seq; a dotted line separates sensitive and resistant cell lines. d, Schematic of the machine learning model training and prediction assessment workflow. RNA-seq data for 17 cell lines were used to generate machine learning models that were trained and refined using empirical testing and then applied to the TCGA Firehose Legacy dataset for TNBC tumours. HVG, highest variable genes; LFC, log-transformed fold change.

maximal GATA3 expression required both EZH2 and AKT inhibitors (Fig. 2f,g). Hi-C data were therefore used to identify *GATA3* enhancer sequences (Extended Data Fig. 7a). Integration of Hi-C and CUT&RUN data showed that *GATA3* enhancer sequences were bound by H3K27me3, which was lost in response to EZH2 inhibition (Fig. 4a and Extended Data Fig. 7b). ATAC-seq analysis further demonstrated that EZH2i and AKTi induced accessibility at multiple regions within this enhancer (Fig. 4a and Extended Data Fig. 7b).

To investigate transcription factor(s) that are involved in GATA3 induction, we first identified proteins with binding sites present in at least one enhancer peak and eliminated those that are not expressed in TNBC lines (the strategy is shown in Extended Data Fig. 7c). We then used single-cell ATAC-seq data from normal mammary glands to identify biologically relevant factors and selected those with motifs that were differentially accessible in luminal versus basal cell populations³⁴. We then focused solely on transcription factors with binding sites that became accessible in response to EZH2/AKTi within the enhancer and also had binding sites within the *GATA3* promoter.

A screen using small interfering RNA (siRNA) was performed to identify which of these transcription factors, when suppressed, could prevent cell death in response to EZH2/AKTi (Fig. 4b). Transcription factors that scored in this assay were then assessed for their ability to suppress GATA3 induction (Fig. 4c). This analysis identified FOXO1 as a critical regulator of the therapeutic response (Fig. 4b) and the most potent regulator of GATA3 induction (Fig. 4c). Importantly, we confirmed that FOXO1 ablation could suppress GATA3 induction and the therapeutic response in multiple cell lines (Fig. 4d,e).

AKT is known to inhibit nuclear localization and transcriptional activity of FOXO1 through phosphorylation³⁵. Indeed, AKTi rapidly triggered FOXO1 dephosphorylation at negative-regulatory Ser256 in multiple TNBC cell lines (Extended Data Fig. 8a). However, we noted that EZH2 inhibitors also induced total FOXO1 expression in these cells (Extended Data Fig. 8a,b). FOXO1 has been reported to be a repressed EZH2 target³⁶, and, consistent with this notion, we found that tazemetostat inhibited H3K27me3 at the *FOXO1* promoter and induced its transcription (Extended Data Fig. 8a–c). These findings suggest that AKT and EZH2 inhibitors both positively regulate FOXO1 in TNBCs. AKT inhibitors permit its transcriptional activity in the nucleus through its dephosphorylation, but EZH2 inhibitors may further enhance these effects by increasing its expression.

While EZH2 inhibitors were sufficient to promote H3K27me3 and open chromatin at the *GATA3* enhancer (Fig. 4a), we predicted that AKT inhibitors would be required for maximal FOXO1 binding. FOXO1 chromatin immunoprecipitation followed by sequencing (ChIP-seq) data were used to precisely define FOXO1-binding sites in the *GATA3* enhancer and promoter (Extended Data Fig. 8d,e). EZH2 and AKT inhibitors cooperatively induced FOXO1 binding at all of these sites (Fig. 4f). Taken together, these data demonstrate that FOXO1 is required for both the induction of GATA3 and the cytotoxic response to EZH2 and AKT inhibitors. Mechanistically, this occurs because tazemetostat opens chromatin at the GATA3 enhancer and increases FOXO1 expression, while ipatasertib is required to induce dephosphorylation of FOXO1 at critical negative regulatory sites.

Death is triggered by BMF upregulation

In addition to the upregulation of luminal markers, ssGSEA revealed that EZH2/AKTi cooperatively induced apoptotic signatures (Extended Data Fig. 9a). BMF, a pro-apoptotic BH3-only protein, was the most significantly and cooperatively upregulated proapoptotic gene and was induced in all sensitive cell lines^{37,38} (Fig. 5a and Extended Data Fig. 9b). As BMF antibodies are not suitable for immunoblot-ting, an HA tag was engineered into the endogenous *BMF* locus using CRISPR technology. We indeed confirmed that the BMF protein was cooperatively upregulated by EZH2/AKTi (Extended Data Fig. 9c). Suppression of BMF by siRNAs prevented the cytotoxic response in multiple cell lines (Fig. 5b, c and Extended Data Fig. 9d, e), demonstrating that its upregulation is also essential for the therapeutic response to EZH2/AKTi.



Fig. 4 | EZH2 and AKT inhibitors drive differentiation by opening chromatin and cooperatively engaging FOXO1. a, Genome browser view of the *GATA3* enhancer with ATAC-seq and CUT&RUN for H3K27me3 data from MDA-MB-468 cells treated with EZH2i and/or AKTi. Chr., chromosome. b, A siRNA screen of candidate transcription factors yields a subset of hits that reduce cell death after combined EZH2/AKTi treatment in MDA-MB-468 cells. c, Relative GATA3 expression compared to siControl + EZH2/AKTi in MDA-MB-468 cells transfected with siRNA against hits from the siRNA screen in b. d, The relative cell numbers after vehicle or EZH2/AKTi treatment after 4 days in SUM149PT or MDA-MB-468 cells transfected with siControl or siFOXO1. *P* values were calculated using unpaired one-tailed heteroscedastic Student's *t*-tests. e, Immunoblot analysis of the MDA-MB-468 samples from d. f, ChIPqPCR analysis of FOXO1 to the *GATA3* enhancer or promoter in MDA-MB-468 cells treated with EZH2i and/or AKTi. Data are technical replicates. Unless noted, data are mean ± s.d. of biologically independent samples.

EZH2/AKTi co-opt involution signals

This finding was intriguing because BMF induction has a critical role in triggering cell death during normal mammary gland involution the programmed destruction of luminal ductal and alveolar cells after the cessation of lactation^{37,38}. Conversely, EZH2 is most highly expressed in ductal cells and alveoli during pregnancy and is substantially reduced during involution¹⁷. Notably, ectopic expression of either *EZH2* or *AKT* in mice has been shown to delay involution, whereas EZH2 or AKT1 ablation accelerates involution^{19,39-42}. Thus, in addition to shifting TNBCs to a more differentiated luminal-like state, we hypothesized that EZH2/ AKTi might be killing TNBCs by activating signals that normally drive the involution of luminal cells.

Involution is regulated by JAK1-STAT3, which triggers a STAT3dependent transcriptional signature that includes BMF^{37,43,44}. Notably, we found that EZH2/AKTi cooperatively induced involution-associated transcriptional signatures in SUM149PT and MDA-MB-468 cells (Fig. 5d). Transcriptional heat maps demonstrate that around three-quarters of involution-associated genes are upregulated in drug treated TNBC cells and that AKT and EZH2 inhibitors both contribute to this response (Fig. 5e and Extended Data Fig. 9f). Moreover, STAT3 ablation prevented the upregulation of BMF (Fig. 5f) and other involution signature genes, as shown by quantitative PCR (qPCR; Extended Data Fig. 9g). [AK1 ablation similarly suppressed BMF expression, which was confirmed at the protein level (Fig. 5g). Finally, AKT and EZH2 inhibitors cooperatively induced STAT3 phosphorylation in TNBC cells (Fig. 5h). The induction of involution signatures and activation of the IAK1-STAT3-BMF pathway were not a general consequence of cell death signals, because concentrations of docetaxel that effectively killed TNBCs did not induce STAT3 phosphorylation, increase BMF expression or stimulate the expression of involution-related genes, at any timepoint (Extended Data Fig. 9h-l). Thus, EZH2/AKTi triggers a transcriptional program in TNBCs that resembles the transcriptional response induced during mammary gland involution. This JAK1-STAT3-driven pathway culminates in the potent upregulation of BMF, which is required for cell death.

Finally, we confirmed that genetic inhibition of STAT3 (Fig. 5i and Extended Data Fig. 9m,n), as well as genetic or chemical inhibition of JAK1 (Fig. 5j,k and Extended Data Fig. 9o), prevented cell death in response to EZH2/AKTi in multiple TNBC lines. Consistent with the cooperative induction of STAT3 phosphorylation and BMF expression, EZH2 and AKT inhibitors were both required to maximally induce STAT3 binding to sites within the *BMF* locus that regulate its expression during involution (Fig. 5I). Thus, AKT and EZH2 inhibitors kill TNBCs by first cooperatively driving luminal differentiation and then together activating an involution-like cell death program.

Involution-related cytokines induce BMF

In considering how EZH2 and AKT inhibitors might cooperatively trigger BMF expression and cell death, we noted that both agents were required for maximal JAK1-STAT3 activation (Fig. 5). During involution, JAK1 is activated by the IL-6 family of cytokines, including LIF, OSM and IL-6 itself. While OSM was not appreciably expressed and LIF was not consistently induced in TNBCs. IL-6 was cooperatively upregulated by EZH2 and AKT inhibitors, which was confirmed by enzyme-linked immunosorbent assay (ELISA; Extended Data Fig. 10a). Genetic ablation of the IL-6 receptor (IL-6R) potently blocked the cytotoxic response to EZH2 and AKT inhibitors in multiple cell lines (Fig. 6a and Extended Data Fig. 10b), which could also be recapitulated by anti-IL-6R antibodies (Extended Data Fig. 10c). Importantly, IL-6R was necessary for the induction of BMF expression (Fig. 6b) and activation of STAT3, as measured by its phosphorylation, in response to EZH2 and AKT inhibitors (Fig. 6c). These results suggest that the cooperativity of EZH2 and AKT inhibitors on the JAK1-STAT3-BMF pathway was conferred at the level of cytokine production.

STING-TBK1 activation induces cytokines

EZH2 inhibitors are known to activate the STING–TBK1 pathway in a variety of tumour types⁴⁵⁻⁴⁷, which can subsequently induce IRF3 and type I interferons or NF-kB and cytokines^{48,49}. Notably, both genetic ablation of *STING* and TBK1 inhibition prevented cell death in response to EZH2 and AKT inhibitors (Fig. 6d) and potently blocked IL-6 production (Fig. 6e and Extended Data Fig. 10d). The STING–TBK1 pathway is activated by CGAS-mediated production of 2'3'-cGAMP, which promotes oligomerization of active STING dimers that subsequently bind to and activate TBK1. Notably, EZH2 inhibition alone induced 2'3'-cGAMP



Fig. 5 | **AKT and EZH2 inhibitors trigger cell death by co-opting signals that normally drive involution. a**, BMF expression (*z* scores) in TNBC cell lines treated with the indicated drugs. **b,c**, The relative change in the cell number (**b**) and immunoblot analysis (**c**) of MDA-MB-468 cells transfected with siControl or siBMF and treated with the indicated drugs. Cells were engineered to express an HA epitope tag knocked into the endogenous *BMF* locus. **d**, ssGSEA *z* scores of mammary gland involution gene signatures in SUM149PT and MDA-MB-468 cells that were treated with the indicated drugs. The box limits show range of data, and the centre lines show the mean values. **e**, Expression of significantly differentially expressed genes in the involution signature from **d** in SUM149PT cells. **f**, *BMF* mRNA expression was measured using RT–qPCR in SUM149PT cells transfected with siControl or siSTAT3 in response to the indicated drugs. **g**, BMF levels (immunoblot of HA knock-in) in MDA-MB-468 cells transfected with

production, which was not enhanced by AKT inhibition (Extended Data Fig. 10e). Consistent with previous observations that EZH2 inhibition can induce 2'3'-cGAMP by derepressing retrotransposable elements and endogenous retroviruses^{47,50-52}, we observed increased expression of endogenous retroviruses in MDA-MB-468 cells treated with tazemetostat alone (Extended Data Fig. 10f). Nevertheless, both EZH2i and AKTi contributed to the formation of STING-TBK1 complexes (Fig. 6f) and subsequent TBK1 activation, as evidenced by autophosphorylation at Ser172 (Fig. 6g). These findings demonstrate that the STING-TBK1 axis is essential for IL-6 production in this context and that both EZH2 and AKT inhibitors promote this response. EZH2 inhibitors do so by activating CGAS/2'3'-cGAMP, whereas AKT inhibitors function further downstream, by enhancing the physical interaction between STING and TBK1. We did not detect any changes in TBK1 or STING phosphorylation using AKT substrate antibodies; however, the precise mechanisms that regulate the NF-kB/cytokine axis of the STING pathway are still unclear^{48,49}. Regardless, these findings provide a defined launching point for future analysis and reveal a role for AKT in this process.

GATA3 and STING agonists reprogram TNBCs

Together, these data suggest that the therapeutic effects of EZH2 and AKT inhibitors require (1) differentiation driven by GATA3 and (2) the induction of BMF through STING. As shown in Fig. 3b, EZH2 and AKT inhibitors do not induce GATA3 in resistant cell lines. However, we also noted that BMF expression minimally increased, if at all (Extended

siControl or siJAK1 and treated with the indicated drugs. The experiment was repeated at least three times. **h**, The relative levels of phosphorylated STAT3 in MDA-MB-468 cells that were treated with the indicated drugs. The experiment was repeated at least three times. **i**, **j**, The relative change in the number of cells transfected with siControl or siSTAT3 (**i**) or siJAK1(**j**) and then treated with the indicated drugs. **k**, The relative change in the number of SUM149PT cells treated with vehicle, a JAK1 inhibitor (itacitinib), AKTi (ipatasertib) and/or EZH2i (tazemetostat). **I**, ChIP–qPCR data of the relative STAT3 occupancy at the *BMF* promoter compared with the IgG (non-specific) control in SUM149PT cells treated with the indicated drugs. For all panels, data are mean ± s.d. of biological independent samples. All experiments are n = 3. *P* values were calculated using unpaired one-tailed heteroscedastic Student's *t*-tests.

Data Fig. 10g). We therefore investigated whether resistant cells could be sensitized by ectopically enforcing both signals. Indeed, overexpression of GATA3 was not sufficient to sensitize cells to EZH2 and AKT inhibitors, and STING agonists alone were also not able to do so (Fig. 6h and Extended Data Fig. 10h). However, cells that both overexpressed GATA3 and were exposed to STING agonists became sensitive to EZH2/ AKTi (Extended Data Fig. 10h), with cooperative effects on cell death readily observed by live-cell imaging (Fig. 6h). As expected, GATA3 expression and STING agonists alone were not sufficient to kill cells on their own (Fig. 6h) because EZH2 and AKT inhibitors are still required to globally open chromatin at GATA3 sites and engage TBK1; however, these findings demonstrate that GATA3 and STING agonists can reprogram resistant TNBCs, therefore conferring sensitivity to EZH2 and AKT inhibitors. A model illustrating this mechanism is shown in Fig. 6i.

Discussion

Standard chemotherapy is not sufficiently effective in advanced TNBC. Even in individuals with PD-L1-positive tumours, who are candidates for chemotherapy combined with immune checkpoint inhibitors, the median overall survival is around 2 years^{53,54}. Thus, developing improved treatments for TNBC is of paramount importance. The success of PI3K inhibitors in HR⁺ tumours has fuelled enthusiasm for targeting this pathway in TNBC^{3,4,10,11}. Nevertheless, clinical trials with PI3K pathway inhibitors in this indication to date have been disappointing⁵⁵. Here we demonstrate how PI3K-pathway inhibitors, specifically AKT



Fig. 6 | **AKT and EZH2 inhibitors trigger involution-related cytokine production by cooperatively activating STING-TBK1 signalling. a**, The relative change in the cell numbers for cells that were transfected with siControl or silL-6R and treated with the indicated drugs. **b**, The relative change in *BMF* expression (RT–qPCR) in SUM149PT cells transfected with siControl or silL-6R and treated with the indicated drugs. **c**, Immunoblot analysis of SUM149PT cells transfected with siRNA against *IL6R* or a control sequence and then treated with vehicle or EZH2/AKTi. **d**, The relative cell numbers in MDA-MB-468 cells transduced with sgControl or sgSTING (left) or treated with TBK1i (right). **e**, The amount of IL-6 (ELISA) relative to the DMSO treatment group in the indicated arms after 8 h. **f**, Immunoprecipitation (IP) of STING or IgG control

inhibitors, can be rendered effective in TNBC, revealing a promising therapeutic strategy for this aggressive disease.

We report that AKT and EZH2 inhibitors synergize and together promote frank tumour regression in multiple TNBC models in vivo. Cell death is preceded by differentiation of TNBCs to a more luminal-like state, which requires both AKT and EZH2 suppression. Importantly, this shift in cell state is required for the therapeutic response; cell death is blocked by inhibiting it and resistant tumour cells do not differentiate in response to EZH2/AKTi.

Mechanistically, EZH2 inhibition opens chromatin at enhancer sequences that regulate GATA3. AKTi then suppresses FOXO1 phosphorylation at critical negative regulatory sites and triggers FOXO1 binding at enhancer and promoter sequences. Once differentiated, EZH2 and AKT inhibitors drive apoptosis by hijacking the same regulatory signals that kill luminal cells during the process of involution. This occurs through the induction of an IL-6–JAK1–STAT3 pathway, which triggers the expression of the pro-apoptotic protein BMF. Moreover, in this therapeutic context, IL-6 induction is regulated by the STING– TBK1 pathway, which is also cooperatively regulated by EZH2 and AKT inhibitors.

Sensitive and resistant cells can be readily distinguished by ATACseq profiles, suggesting important baseline differences in their epigenetic state. This observation is consistent with the inability of resistant cells to differentiate into a more luminal-like state. Fortunately, a machine learning approach yielded a transcriptional classifier that can be used to select individuals with tumours that are most likely to respond. Moreover, we found that resistant cells can be reprogramed by ectopically enforcing differentiation and cell death signals through GATA3 expression and STING agonists, suggesting that resistance is reversible. in MDA-MB-468 cells treated with the indicated drugs for 8 h followed by immunoblotting using the indicated antibodies. **g**, Immunoblot analysis of MDA-MB-468 cells that were treated with EZH2i and/or AKTi using the indicated antibodies. **h**, Live-cell imaging of SUM159PT cells (resistant) transduced with *GFP* or *GATA3* overexpression constructs treated with EZH2/AKTi and/or STING agonist ADUS100 (STINGag). **i**, The mechanism of action of combined EZH2 and AKT inhibitors to induce cell death and tumour regression in TNBC. The diagram was created using BioRender. For all panels, data are mean ± s.d. of biologically independent samples. All panels showing immunoblots were repeated at least three times. For all experiments, *n* = 3. *P* values were calculated using unpaired one-tailed heteroscedastic Student's *t*-tests.

Together, these studies suggest that EZH2 functions as an epigenetic insulator in TNBCs, shielding tumour cells from luminal differentiation and cell death, by suppressing the expression of GATA3 and BMF. However, AKT has an equally important role in this process, as EZH2 and AKT suppression are both required for maximal upregulation of these genes and cell death through mechanisms described here. Notably, EZH2 and AKT (suppression) have independently been implicated in mammary gland involution in mice, and the expression of both proteins plummets at the onset of involution^{17,19,39,40,42}. However, the precise mechanisms by which EZH2 and/or AKT function in this context are not well understood. In TNBCs, we have shown that these proteins cooperatively regulate the activation of IL-6, JAK1, STAT3 and BMF-key components that regulate cell death during involution. However, we cannot exclude the possibility that involution genes might be generally triggered by some apoptotic inducers and/or that other apoptotic genes not normally involved in involution may contribute to the response. Nevertheless, JAK1, STAT3 and BMF are three components that are essential for cell death in both contexts.

It will be interesting to determine whether EZH2 and AKT inhibitors synergize in other tumour types and, if so, whether identical or other tissue-specific mechanisms are engaged. Certainly, any shift in cell state, if observed or required, would probably be mediated by tissue-specific factors and/or could be instructed by tissue-specific epigenetic states. It will also be equally interesting to investigate whether this paradigm extends beyond these two proteins and their inhibitors in breast cancer and whether epigenetic agents may more broadly sensitize tumours to canonical oncogenic inhibitors. If so, deconstructing how specific epigenetic and oncogenic pathways converge will be critical for the rational design of future combinatorial strategies. Regardless, the data presented here provide compelling support for the development of clinical trials exploring the combined effects of AKT and EZH2 inhibitors in TNBC.

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Methods

Cell counting

To measure cellular proliferation and cytotoxicity, manual cell counting experiments were conducted. On day -6. cells were seeded at 40% confluency in 10 cm dishes. On day -5, cells were treated with DMSO or tazemetostat. On day -3, cells were passaged 1:2 and maintained in tazemetostat or DMSO. On day -1, cells were seeded at 250,000 cells per well into six-well tissue culture dishes for counting (enough for day 0 and for day 4 counts) and 625,000 cells per 6 cm dish for protein lysate collection. For cell counting assays including siRNA, cells were transfected on day -1 and incubated with siRNA for 6-8 h before seeding. On day 0, starting cell counts were measured using a haemocvtometer and the remaining cells were dosed with DMSO, ipatasertib. tazemetostat or the combination. On day 1, protein lysates were collected. On day 4, remaining cells were counted using a haemocytometer. The log₂-transformed fold change in cell number was calculated by normalizing day 4 cell count values to day 0 cell count values. For days -6 to -1, cells were cultured in appropriate medium containing 10% FBS. For days 0 to 4, cells were cultured in appropriate medium containing 2% FBS. All of the experiments were completed at least three times. Statistical methods were not used to pre-determine sample size. Blinding was not conducted. Cells were seeded from a master solution and allocated across all compared conditions to control for seeding and population differences.

IncuCyte live-cell imaging

Live-cell imaging was completed using IncuCyte live-cell imaging. After 5 days of pretreatment with tazemetostat or DMSO, cells were seeded at 5,000 cells per well in 96-well plates. Then, 24 h after seeding, cells were treated with tazemetostat and/or ipatasertib in medium containing NucLight Rapid Red Reagent (Sartorius, 4717) to label nuclei and Cytotox Green (Sartorius, 4633) to label cytotoxic cells. Plates were placed inside the IncuCyte machine and images were acquired every 2 h for 5 days. The percentage of cytotoxic cells was determined using the integrated IncuCyte software by quantifying the overlap of NucLight- and Cytotox-green-positive cells divided by the total number of NucLight-positive cells. Four images per well were taken and each condition was seeded in duplicate. All of the experiments were completed at least three times.

Synergy score analysis

To measure synergistic interactions between EZH2 inhibitor tazemetostat combined with PI3K–AKT pathway inhibitors, cells were seeded in 96-well white flat bottom plates at 5,000 cells per well. For each condition, two technical replicates were seeded per combination treatment. After 5 days of pretreatment with EZH2 inhibitor, cells were dosed with PI3K–AKT pathways inhibitors. Drugs were added at the following concentrations: EZH2 inhibitor tazemetostat (0, 1.25, 2.5, 5, 10 μ M), and AKT inhibitor lpatasertib (0, 0.625, 1.25, 2.5, 5, 10 μ M). After 96 h of treatment, cell viability was quantified using CellTiter-Glo (Promega, G9291) and normalized to DMSO to calculate the inhibitory response. SynergyFinder was then used to calculate the synergy score using the Gaddum's non-interaction model–highest single agent (HSA) model, where a value of greater than 10 indicates a synergistic interaction⁵⁶.

Orthotopic xenografts

All mouse work was done in compliance with the Institutional Animal Care and Use Committee (IACUC) at Brigham and Women's Hospital. The maximum tumour size was 20 mm in one dimension of the tumour. SUM149PT and MDA-MB-468 xenografts were generated by injecting 3×10^6 cells using a 1:1 ratio of DMEM/F12 and Matrigel (Corning, 356231) into the fourth inguinal mammary fat pad of nude mice. For the organoid allograft, organoids were generated from *Trp53*^{fUfl} mice and

cultured as previously described^{23,24} and 50,000 cells were implanted into the fourth inguinal mammary fat pad of nude mice. For SUM149PT and MDA-MB-468 xenograft studies, two tumours per mouse were implanted. For the organoid allograft, MDA-MB-468 xenograft survival study and PDXs, one tumour per mouse was implanted. Tumour fragments of HCI-025 and HCI-004 were implanted into NSG mice. Tumours arose after 2 weeks and were enrolled when they reached 100-250 mm³. The tumour volume was measured using vernier callipers with the formula (width \times width \times length \times 0.52). For the treatment schedule, mice were pretreated with tazemetostat or vehicle for 7 days before being treated with vehicle (ipatasertib vehicle + tazemetostat vehicle), ipatasertib (ipatasertib + tazemetostat vehicle), tazemetostat (ipatasertib vehicle + tazemetostat) or combination (tazemetostat + ipatasertib). Ipatasertib was dosed at 50 mg per kg once per day through oral gayage. Ipatasertib was prepared in 0.5% methylcellulose, viscosity 4,000 cP, with 2% Tween-80 at pH 7.0 and kept at 4 °C for up to 1 week. Tazemetostat was dosed at 250 mg per kg twice a day through oral gavage. Tazemetostat was prepared in 0.5% methylcellulose containing 0.1% Tween-80 and kept at 4 °C for up to 4 weeks. PDXs HCI-025 and HCI-004 were prepared and passaged as previously described^{25,57}. Sample size was pre-determined: we calculated that if we used 10 mice per treatment group, we would have 87% power to detect a 50% reduction in tumor size, and every effort was made to reach that sample size. Animals were randomized into treatment arms. Blinding was not conducted. The temperature in rooms housing mice was kept stably between 68 and 75 °C. The humidity range of rooms housing mice was kept stably at 35-65%, with 50% humidity being considered optimal. Animals experienced 12 h of light during the day and 12 h of dark during the night with a light intensity between 100-250 lux. Mice were offered diet ab libitum in food hoppers hung from the top of the cage with Pico 5052 Irradiated Rodent Chow or Pico 5058 Irradiated Mouse Chow. Mouse were offered water ad libitum. Mice were housed in Allentown ventilated cages with bedding approximately 1/2 inch in depth with Alpha-blend or crinkled paper (nude mice received paper-based bedding only). Mice were offered gnawing enrichment in the form of nestlets or popsicle sticks (nude mice were offered only popsicle sticks). Raw animal data are provided in Supplementary Data 2.

Cell lines

Cell lines were purchased directly from ATCC or authenticated using STR analysis (Labcorp). SUM149PT and SUM159PT cells were cultured in DMEM/F12 (Gibco, 11330-057), 293T, RPE1, MDA-MB-157, MDA-MB-231, MDA-MB-436 and MDA-MB-468 cells were cultured in DMEM (Corning, 10-013-CV), and BT20, BT549, DU4457, HCC38, HCC70, HCC1143, HCC1187, HCC1395, HCC1806, HCC1937 and HCC2157 cells were cultured in RPMI (Corning, 10-040-CV), IMR90 and BJ fibroblast cell lines were cultured in EMEM (Thermo Fisher Scientific, 10-009-CV). All media were supplemented with 10% FBS, and 1× concentration of penicillinstreptomycin–glutamine (Gibco, 10378016). *TP53^{-/-}* organoids were cultured as previously reported^{23,24}. MCF10A cells were cultured as previously reported⁵⁸. No commonly misidentified cell lines were used in this study. Cell lines were routinely found to have no mycoplasma contamination using Lonza MycoAlert PLUS Mycoplasma Detection Kit.

Crystal violet

Cells were seeded at 50,000 cells per well into 12-well plates and then stained with crystal violet at the indicated times. In brief, after the medium was removed, wells were washed with PBS three times before fixation with 2% formaldehyde (1:2 dilution of Formalde-Fresh Solution; SF94-4) for 15 min a room temperature. After fixation, wells were washed with PBS once before staining with 0.025% crystal violet (Sigma-Aldrich, C6158) for 2 h. After staining, wells were washed with water four times before air drying and imaging using a standard scanner.

Immunohistochemistry

Tumours were collected for immunohistochemistry 4 h after final dose of drug treatment by removing tumour and fixing in Formaldehyde-Fresh (Thermo Fisher Scientific, SF94-4) for 24 h. After 24 h, tumours were stored in 70% ethanol before sectioning and analysis. For haematoxylin and eosin staining, sectioning and staining was performed at the Harvard Medical School Rodent Histopathology Core. For Ki-67 and EZH2 (BD Bio, 612667) immunohistochemistry, sectioning and staining was performed at the Brigham & Women's Hospital Pathology Core.

siRNA and CRISPR

Cells were incubated for 6-8 h with 0.1 uM siRNA constructs using a 1:400 dilution of Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, 13778075) in antibiotic-free medium. siRNA ON-TARGETplus pools were purchased from Horizon Biosciences: control (D-001810-10-50), GATA3 (L-003781-00-0005), JAK1 (L-004393-00-0010), BMF (L-003145-00-0005), STAT3 (L-003544-00-0005), IL-6R (007994-00-0005), BACH2 (L-009787-00), HSF1 (L-012109-02), MEF2D (L-009884-00), NFIA (L-008661-00), NFIX (L-009250-00), PRDM1 (L-009322-00), RELB (L-004767-00), ZNF143 (L-013965-00), ZNF354C (L-014199-02), BACH1 (L-007750-00), ELF3 (L-016080-00-0005), FOS (L-003265-00), FOXO1 (003006-00-0005), FOXO3 (003007-00-0005), HOXC11 (L-017602-00-0005), HOXD11 (L-013095-00-0005), HOXD12 (L-013096-02-0005), JUN (L-003268-00), MAFK (L-008580-00), SIX2 (L-017024-00), ETV1 (L-003801-00), ETV4 (L-004207-00), FOSL1 (L-004341-00), FOSL2 (L-004110-00), HOXC10 (L-017601-00), JDP2 (L-008321-01), JUNB (L-003269-00), JUND (L-003900-00), MAFF (L-003903-00), MAFG (L-009109-00), MAFK (L-008580-00), MITF (L-008674-00), MXI1 (L-009947-00), PBX3 (L-020121-00), PKNOX1 (L-021413-00), SIX1 (L-020093-00), SREBF2 (L-009549-00), TBX1 (L-012195-00), USF1 (L-003617-00), USF2 (L-003618-00) and VDR (L-003448-00). shRNA constructs in the pLKO.1 backbone against non-targeting and GATA3 (TRCN0000019299) were purchased from Sigma-Aldrich. sgRNA constructs in the pLentiCRISPR-v2 vector expressing Cas9 against control, STING and GATA3 were purchased from GenScript.

Inhibitors

For all assays except synergy assays, tazemetosat was used at 5 μ M (S7128, Selleckchem), ipatasertib was used at 5 μ M (S2808, Selleckchem), BYL719 was used at 5 μ M (S2814, Selleckchem), GDC0077 was used at 5 μ M (S8668, Selleckchem), GDC0941 was used at 3 μ M (S1065, Selleckchem), MAK683 was used at 5 μ M (S8983, Selleckchem) and itacitinib (JAK1i) was used at 500 nM (S7812, Selleckchem). TBK1 inhibitor GSK8612 was used at 16 μ M (S8872, Selleckchem). Anti-IL-6R antibody is tocilizumab and was supplemented at 50 μ g ml⁻¹ (A2012-5MG, Selleckchem). STING agonist ADU-S100 (MIW815) was supplemented at 50 μ M (CT-ADUS100, Chemietek). Docetaxel was supplemented at 2 nM or 10 nM (S1148-10MM/1ML, Selleckchem).

ELISA

Human IL-6 (Thermo Fisher Scientific, KHC0061) and 2'3'-cGAMP (Cayman Chemical, 501700) were detected with ELISAs according to the manufacturer's instructions. Samples for IL-6 (conditioned media) or 2'3'-cGAMP (protein lysates) were collected 24 h after addition of AKTi and/or TBK1i. Values represent the average of three or four biological replicates normalized to the DMSO sample.

GATA3 expression plasmid

GATA3 ORF was purchased from Addgene (plasmid 81902) in pDONR221 vector⁵⁹ and cloned into pHAGE-puro expression vector (Addgene plasmid 118692) using LR clonase II (11791020; Thermo Fisher Scientific) according to the manufacturer's specifications. GATA3 overexpression

was enforced in SUM159PT cells by lentiviral transduction and puromycin selection (1 μg ml $^{-1}$) for 72 h.

Western blotting

Protein was collected after 24 h of vehicle, single agent or combination treatment. Cells were washed with PBS (806544-500ML, Sigma-Aldrich) and then boiling 1% SDS lysis buffer (1% SDS (15553-035, Invitrogen), 10 mM Tris-HCl pH 7.5 (77-86-1, Sigma-Aldrich), 100 mM NaCl (S5586, Sigma-Aldrich)) was directly added to plates. Plates were scraped and protein lysate was sheared using 20 G needle 5-6 times. Proteins were boiled at 95 °C for 10 min and centrifuged at maximum speed for 3 min. The protein concentration was determined using the BCA quantification method (23222, Bio-Rad). Proteins were run in SDS-PAGE gels (4561084, Bio-Rad) and transferred to Immobilon PVDF membranes (IPVH00010, Sigma-Aldrich). Membranes were blocked with 5% milk in TBST for 1 h before incubation with primary antibodies overnight. The membranes were incubated with HRP-conjugated secondary antibody corresponding to the species of the primary antibody. HRP signal was measured using autoradiography film or by BioRad Chemidoc. Antibodies for western blotting were as follows: H3K27me3 (9733S, Cell Signaling Technologies, 1:1,000), H3 (4499S, Cell Signaling Technologies, 1:1,000), PRAS40 (2610S, Cell Signaling Technologies, 1:1,000), pPRAS40 (2997S, Cell Signaling Technologies, 1:1,000), GAPDH (2118S, Cell Signaling Technologies, 1:1,000), GATA3 (558686, BD BioSciences, 1:250), pSTAT3 (sc-8059, Santa Cruz, 1:500), STAT3 (9139, Cell Signaling Technologies, 1:1,000), JAK1 (3332S, Cell Signaling Technologies, 1:1,000), P27 (sc-528, Santa Cruz, 1:1,000), FOXO1 (9454, Cell Signaling Technologies, 1:1,000), FOXO1-pS265 (9461, Cell Signaling Technologies, 1:500), TBK1 (3504S, Cell Signaling Technologies, 1:1,000), TBK1-pS172 (5483S, Cell Signaling Technologies, 1:1,000), STING (13647S, Cell Signaling Technologies, 1:1,000), anti-rabbit secondary (111-035-144, Jackson ImmunoResearch, 1:5,000), anti-mouse secondary (115-035-166, Jackson Immuno-Research, 1:5,000). All uncropped images of immunoblots are provided in Supplementary Data 1.

STING-TBK1 co-IP

Cells were treated with the indicated drugs and then collected after 8 h of treatment by washing with PBS and scraping. Pelleted cells were lysed using EBC lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.5% NP-40, 1:10,000 β mercaptoethanol, 0.5 mMEDTA) supplemented with protease inhibitors (Sigma-Aldrich, 11836153001) and phosphatase inhibitors (Sigma-Aldrich, 4906837001). Cell lysates were incubated on ice for 20 min and then cleared by centrifugation at 16,000*g* for 20 min. Protein extracts were quantified using BCA reagent and then equal quantities were incubated with STING (Cell Signaling, 13647S) or IgG (Cell Signaling, 2729S) antibody and magnetic protein A/G beads (Pierce) overnight with end-over-end agitation at 4 °C. Beads were washed five times with EBC lysis buffer before immunoprecipitated proteins were eluted using SDS sample buffer and analysed by western blotting (described above).

RNA-seq

RNA was isolated after 8 or 24 h of treatment using the RNeasy Plus kit (74134, Qiagen). RNA was sequenced at the Dana-Farber Cancer Institute Molecular Biology Core Facility (SUM149PT, MDA-MB-468) or Novogene (MDA-MB-468 docetaxel experiment, HCC38, HCC1395, HCC1937) using the Illumina NextSeq 500 system. Raw data were mapped to the Hg19 (SUM149PT, MDA-MB-468) or hg38 (MDA-MB-468 docetaxel experiment, HCC38, HCC1395, HCC1397) genome using STAR and count files were made using HTSeq^{60,61}. DESeq2 was used to normalize counts (mean-ratio method), calculate total reads and determine differentially expressed genes⁶². Volcano plots were generated using EnhancedVolcano and heat maps were generated using pheatmap^{63,64}. DESeq2 was used to determine differentially

expressed genes for public RNA-seq raw counts collected from CCLE database. Volcano plots were generated using the ggplot2 package in R and heat maps were generated using ComplexHeatmap package in R.

ATAC-seq

ATAC-seq data were sequenced using the Illumina NovaSeq 6000 system. Using the ENCODE pipeline (https://www.encodeproject.org/ atac-seq/), raw sequencing FASTQ data were read and aligned to the hg38 reference genome. MACS2 was used to identify peaks indicating open chromatin region. We filtered out non-reproducible peaks by selecting peaks that were detected in replicates of each condition for each cell line, then peaks were merged for overlapping region across all conditions and samples using the GenomicRanges package in R. The consensus peaks were annotated using ChIPseeker package in R. Using featureCounts in Rsubread package in R, we counted all reads over the consensus peak regions, then performed differential accessible peak analysis using DESeq2. Motif discovery and analysis was performed using RGT-HINT. Transcription-factor-binding sites were identified with JASPAR motifs. Transcription factor activity scores were estimated based on the transcription factor footprint profiles. We ranked genes based on chromatin accessibility differences in promotor. If multiple peaks were annotated to single gene, we estimate the major changes by taking maximum/minimum log-transformed fold changes if <80% of peaks are more open/closed. The ranked genes were used for GSEA using the fgsea package in R. Gene sets were collected from the entire MSigDB using the msigdbr package in R.

Machine learning model

For the initial training set, TPMs of RNA-seq data for 17 cell lines (10 sensitive, 7 resistant) were collected from the CCLE dataset and standardized using z-score normalization. Feature selection is important for machine learning training as too many irrelevant features can interrupt model training (for example, overfitting). We therefore selected subset of genes by ranking them in terms of its variance or log-transformed fold change. First, we selected genes that were differently expressed between sensitive and resistant cell lines with FDR <0.05. We then ranked the genes based on their variance and selected the top 50, 100 and 200 highly variable genes (represented as top 50, top 100, and top 200, respectively). We also selected genes based on a cut-offs for the log-transformed fold change in sensitive versus resistant of 2 and 5, that is, |LFC| > 2 and |LFC| > 5. The models were trained using two different algorithms, RF and SVM, on the training sets with the subset of genes, then evaluated by its accuracy. We validated the models using leave-one-out cross validation. During the validation, the dataset is divided into N subsets where N is the total number of cell lines. Each subset includes N-1 cell lines by removing one cell line as the validation set. For each subset, we performed differential expression analysis, ranked differentially expressed genes, defined a subset of genes based on its rank, trained machine learning models on the subset then tested the models using the validation cell line that was not used in the entire process at all. This was repeated N times by removing one cell line each time. In total, we have $2 \times 5 \times N$ models as we use two algorithms (RF, SVM) and five gene sets (top 50, top 100, top 200, |LFC| > 2 and |LFC| > 5). Cross validation accuracy is calculated by the number of correctly predicted unused cell line/N. On the basis of the validation and test accuracy, we selected the best model (RF, |LFC | > 5), then retrained the model using 17 cell lines. We used the final model to predict the sensitivity of TNBC tumours in the TCGA database. Similarly, TPMs of RNA-seq data were obtained then scaled using z-score normalization. Feature selection, including differential expressions and rank genes, were implemented in R. All of the machine learning methods, including training, validation, and testing, were implemented using the scikit-learn library in Python.

Copy-number analysis

To measure copy number alterations in *TP53^{-/-}* organoid allograft tumours, we completed ultra-low-pass whole-genome sequencing with the Dana-Farber Cancer Institute Molecular Biology Core Facility. Genomic DNA was isolated from a tumour from an untreated tumour-bearing mouse, fragmented and sequenced. Approximately 5 million library reads were analysed for copy variation at 100 kb resolution. We used CNVkit pipeline to process the germline sequencing raw data. The scatter plot was generated using the CNVkit library in Python.

ssGSEA and GSEA

ssGSEA was performed using GenePattern (https://www.genepattern. org/)^{65,66}. GSEA was performed using software available online (http:// www.gsea-msigdb.org/)^{65,67}. Gene sets are published on the MSigDB for hallmark apoptosis (M5902, HALLMARK_APOPTOSIS)⁶⁸, mammary stem cells (M2573, LIM_MAMMARY_STEM_CELL_UP)²⁶ and mature luminal mammary cells (M2578: LIM_MAMMARY_LUMINAL_MATURE_UP)²⁶. Mammary involution gene set was derived from a previous study that identified genes upregulated in involution that were dependent on JAK1 expression in the mammary gland³⁷.

TNBCtype analysis

Molecular subtyping of samples using RNA-seq values was performed using the TNBC type tool^{27,69}. Correlation coefficient with most significantly altered subtypes was reported for each cell line.

BioRender

The model presented in Fig. 6i was created using BioRender (license number IY273VQW9J).

ChIP-qPCR

MDA-MB-468 cells were plated at 1.2 \times 10 6 in 15 cm plates and treated the same as for cell counting and immunoblotting. After 24 h of vehicle, single agent or combination treatment, cells were collected in native medium. Cells were cross-linked first with 2 mM DSG (20593, Pierce) for 45 min at room temperature followed by 1% formaldehyde for 10 min at 37 °C before quenching with 0.125 M glycine for 5 min. Chromatin was isolated by first incubating with buffer 1 (50 mM HEPES-KOH pH 7.5 (15630-080, Thermo Fisher Scientific), 140 mM NaCl (S5586, Sigma-Aldrich), 1 mM EDTA pH 8 (15575-038, Invitrogen), 10% glycerol (G5516, Sigma-Aldrich), 0.0033% NP-40 (493015, Calbiochem) and 0.25% Triton X-100 (T8787, Sigma-Aldrich)), then resuspending the pellet in buffer 2 (10 mM Tris-HCl pH 8 (15567-025, Invitrogen), 200 mM NaCl, 1 mM EDTA pH 8 and 0.5 mM EGTA pH 8), and finally resuspending the cell pellet with buffer 3 (10 mM Tris-HCl pH 8,100 mM NaCl, 1 mM EDTA pH 8, 0.5 mM EGTA pH 8, 0.1% sodium deoxycolate (D6750, Sigma-Aldrich) and 0.5% N-laurosarcosine (L7414, Sigma-Aldrich)). Isolated chromatin was then sonicated for 30 cycles of 30 s on, 30 s off using a Diagenode BioRuptor Plus sonicator on 'high'. Soluble chromatin was immunoprecipitated with FOXO1 (2880, Cell Signaling Technologies), IgG (2729S, Cell Signaling Technologies) or STAT3 (9139, Cell Signaling Technologies) antibodies overnight. Immunoprecipitated chromatin was captured with magnetic protein A/G beads (88803, Pierce). Immunoprecipitated chromatin was washed with low-salt buffer three times (0.1% SDS, (15553-035, Invitrogen), 1% Triton X-100, 2 mM EDTA pH 8, 20 mM Tris-HCl pH 8 and 150 mM NaCl), high-salt buffer three times (0.1% SDS, 1% Triton X-100, 2 mM EDTA pH 8, 20 mM Tris-HC-l pH 8, 500 mM NaCl), LiCl buffer three times (0.25 M LiCl (62476, Sigma-Aldrich), 1% NP-40, 1 mM EDTA pH 8, 10 mM Tris-HCl pH 8, 1% sodium deoxycholate) and TE buffer (10 mM Tris-HCl pH 8, 10 mM EDTA pH 8) before elution in elution buffer (50 mM Tris-HCl pH 8, 10 mM EDTA, 1% SDS). Eluted chromatin was purified using the QIAquick PCR purification kit (28106, Qiagen). Relative abundances of BMF and GATA3 promoters in immunoprecipitated chromatin was

measured using qPCR using PerfeCTa SYBR Green SuperMix Reaction Mixes (95054-500, QuantaBio) and primers listed below. The percentage input values were calculated for each immunoprecipitation. Fold change enrichment values were calculated by normalizing to the percentage input of the appropriate IgG control for each immunoprecipitation.

HA knock-in BMF

An N-terminal HA-tag was introduced into the endogenous locus of BMF as previously described⁷⁰. The crRNA (5'-TTGCCCCCTCAC AGGAGAGA-3') was hybridized with Alt-R CRISPR-Cas9 tracRNA (IDT, 1073190) at 95 °C for 5 min, at an equimolar ratio (0.375 nmol). The mix was cooled down to room temperature on the benchtop for 10 min before adding 2 ul of Alt-R S.p. Cas9 Nuclease V3 (IDT. 1081059) and 5 µl of 100 µM single-stranded donor oligonucleotide (5'-GCTGAGGGGGGCAGTCCAGTAGGCTCTGGGCAAACAGG TCAGCAGAGAGCAAGCTCCCGGGTTGGGTCACCGGCTCCCCATCCT CTGGTTGGAACACATCATCCTCCAGCTCCTCCACACACTGAGATGGCT CAGCGTAATCTGGTACGTCGTATGGGTACATCTCTCCTGTGAGGGGGC AACGCAGGCATCTGGGCTGCT-3'). The mix was incubated for 20 min at room temperature and added to 1.5 million (MDA-MB-468) or 1 million (HCC1395) cells resuspended in 100 µl of SF Cell Line Nucleofector solution (Lonza, V4XC-2012). Cells were then transferred to a cuvette and nucleofected using the EO-117 program in a 4D-Nucleofector X Unit (Lonza).

CUT&RUN

CUT& RUN was performed as previously described⁷¹. In brief, 500,000 nuclei from MDA-MB-468 cells treated with DMSO, 5 µM ipatasertib, 5 µM tazemetostat or a combination of both drugs were isolated using nuclear extraction buffer (20 mM HEPES pH 7.9, 10 mM KCl, 0.1% Triton X-100, 20% glycerol and 1 mM MnCl₂). Nucleus samples were then immobilized to Biomag Plus Concanavalin A (Con A)-coated magnetic beads (Bangs laboratories) that were activated by washing three times with cold bead activation buffer (20 mM HEPES pH 7.9, 10 mM KCl, 1 mM CaCl₂, 1 mMMnCl₂). ConA bead/cell mixtures were resuspended in cold antibody buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 0.5 mM spermidine, 1× Roche cOmplete, Mini, EDTA-free protease inhibitor, 0.01% digitonin, 2 mM EDTA), then incubated with 0.5 µg primary antibodies: H3K27me3 (CST, 9733) and H3K4me3 (CST, 9733) or IgG (Epicypher) overnight in a 4 °C cold room. Unbound antibodies were washed three times each with cold digitonin buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 0.5 mM spermidine, 1× Roche cOmplete, Mini, EDTA-free protease inhibitor, 0.01% digitonin). ConA bead/cell mixtures were then resuspended in 50 µl cold digitonin buffer and incubated with our homemade pAG-MNase in a 4 °C cold room for an hour on a nutator. Unbound pAG-MNase was washed three times with cold digitonin Buffer. MNase was activated by addition of CaCl₂ and incubated in a 4 °C cold room for 30 min on a nutator to cleave and release antibody-bound chromatin. The reaction was stopped by adding cold stop buffer (340 mM NaCl, 20 mM EDTA, 4 mM EGTA, 50 μg ml⁻¹ RNase A, 50 μg ml⁻¹ glycogen, 1 pg μ l⁻¹*E. coli* spike-in DNA). Cleaved chromatin was then released by incubating at 37 °C for 10 min. CUT&RUN enriched DNA in the supernatant can be collected using magnetic beads and purified using the Monarch PCR and DNA cleanup kit (NEB T1030L). CUT&RUN libraries were prepared with 10 ng CUT&RUN DNA using NEBNext Ultra II DNA library prep kit (NEB), according to manufacturer's protocol. Libraries were sequenced on the Illumina NextSeq 2000 system, 2 × 50 bp paired-end reads.

Paired-end fastq files were aligned to hg38 reference genome using Bowtie2 with the settings '--very-sensitive --no-mixed --no-discordant --phred33 -110 -X 700'⁷². Sequencing reads were also aligned to the *E. coli* genome to map spike-in reads. For spike-in normalization, the total number of mapped reads to *E. coli* genome was used to calculate normalization factor for CUT&RUN samples. SAM files were converted to BAM files using samtools⁷³. Bigwig files were generated from BAM files using Deeptools⁷⁴. Genome browser tracks of big files were generated using the Integrative Genomics Viewer (IGV)⁷⁵. Peaks were called using MACS2 using callpeak function with '-f BAME -keep-dup1-q0.05' and IgG was used as a control⁷⁶. DeSeq2 analysis from DiffBind R package with option 'spikein=True' was used to compare differential binding between conditions (FDR < 0.1)⁷⁷.

RT-qPCR

RNA was isolated using the RNeasy Plus kit (74134, Qiagen). RNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (4368814, Thermo Fisher Scientific). qPCR was completed using PerfeCTa SYBR Green SuperMix Reaction Mixes (95054-500, QuantaBio) and the primers listed below. C_q values were normalized to *STAU1* as a reference gene.

CyCIF analysis

Human breast cancer tissue array BRC482 was purchased from Pantomics and consists of 16 cases, with one normal breast tissue core paired with two tumour tissue cores from each patient. FFPE sections of PDX samples and a tissue microarray were prepared and stained with a 23-plex and 33-plex antibody panel, respectively, using tissue-based cyclic immunofluorescence (CyCIF) as previously outlined. Baking and dewaxing: to prepare the samples for antibody staining, tissue sections on glass slides underwent an automated process facilitated by the Leica Bond RX machine. The protocol started with a 30 min baking step at 60 °C. Subsequently, the slides were subjected to dewaxing at 72 °C in BOND Dewax Solution, followed by an antigen-retrieval step conducted at 100 °C for 20 min using BOND Epitope Retrieval Solution 1 (ER1). Photo-bleaching and autofluorescence reduction: after the baking and dewaxing steps, the slides were immersed in a bleaching solution (4.5% H₂O₂, 20 mM NaOH in 1× PBS) and exposed to LED light for 1 h to mitigate autofluorescence. Excess bleaching solution was removed by washing the slides six times with 1× PBS (10-15 s each). Subsequently, the slides were incubated overnight at 4 °C in darkness with secondary antibodies diluted in SuperBlock Blocking Buffer (1:1,000; Thermo Fisher Scientific, 37515). After this incubation, the slides were washed six times with 1× PBS and were photobleached again for 1 h. Antibody staining, coverslip mounting and imaging: for each CyCIF cycle, the samples were incubated overnight at 4 °C in the absence of light with Hoechst 33342 (1:10.000: Thermo Fisher Scientific, 62249) for nuclear staining. Simultaneously, primary antibodies were introduced, which were either conjugated or unconjugated, diluted in SuperBlock Blocking Buffer (Thermo Fisher Scientific, 37515). In cases in which primary antibodies were unconjugated, subsequent incubation with secondary antibodies was performed at room temperature for 1 h in the dark. After staining, the slides underwent a series of six 10 s washes with 1× PBS, and then they were mounted with 24×60 mm coverslips, using $80-100 \mu$ l of 50% glycerol, and left to dry at room temperature for 30 min. Once coverslipped, automatic imaging was conducted using the RareCyte Cytefinder II HT system with the following channels: UV, cy3, cy5 and cy7. Imaging parameters remained consistent as follows: binning: 1×1 for PDX whole-slide images; binning 2 × 2 for the TMA; objective: ×20; numerical aperture: 0.75; resolution: 0.325 mm px⁻¹. Image exposures were fine-tuned for each channel to prevent signal saturation but were maintained uniformly across samples. Between CyCIF cycles, the coverslips were demounted by immersing slides in containers of 1× PBS (5 slides per container) for 10 min at room temperature. Before initiating the subsequent antibody staining cycle, slides were photobleached for 1 h to deactivate the fluorophores and washed again in PBS six times to remove residual bleaching solution. The acquired images were processed using the Docker-based NextFlow pipeline MCMICRO. Initially, raw images from the Cytefinder II HT underwent illumination correction via the BaSic module. Subsequently, they were stitched and registered using the ASHLAR module, resulting in the generation of

assembled OME.TIFF files for each slide. Full code is available at GitHub (https://github.com/labsyspharm/mcmicro).

Antibodies were as follows: CK14 (Abcam, ab77684, LL002, conjugated to FITC, 1:500), CK8 (eBioscience, 11-9938-80, LP3K, conjugated to FITC, 1:400), pan-CK (eBioscience, 53-9003-82, AE1/AE3, conjugated to AF488, 1:1,000), EZH2 (CST, 45638, D2C9, conjugated to AF647, 1:100), cPARP (CST, 6987S, D64E10, conjugated to AF647, 1:100), E-cadherin (CST, 9835S, 24E10, conjugated to AF647, 1:600), anti-rabbit IgG (Invitrogen A-31572, conjugated to AF555, 1:1,000) and anti-mouse IgG (Invitrogen, A-31571, conjugated to AF647, 1:1,000).

ERV expression

To count reads for ERVs, ERV locus information was collected from the ERVmap database⁷⁸. Salmon (v.0.14.1)⁷⁹ was used to create a customized reference transcriptome set that includes human genome transcriptome (hg38) and ERVmap database. Using the database, RNA-seq data of MDA-MB-468 cells treated with DMSO or EZH2i tazemetostat were quantified by Salmon. DESeq2 was used for counts normalization and differential expression analysis.

Oligonucleotides

Primers for RT-qPCR were as follows in 5'-3' orientation: STAU1 (fwd: GGATGAGTTCAGGATGCCTTAT, rev: GGTGTGATGTCCTTGACTAACT), BMF (fwd: ACTTCAGCTCTTCCCTCTCA, rev: GAGTCTGGGTAGCTT TGTCTTC), GATA3 (fwd: CTCATTAAGCCCAAGCGAAG, rev: GTCTG ACAGTTCGCACAGGA), FOS (fwd: GTCTTCCTTCGTCTTCACCTAC, rev: GAGTCAGAGGAAGGCTCATTG), CYBRD1 (fwd: AGATCCTGCATACA GTACATTCC, rev: CATTGCGGTCTGGTGACTAT), PDK4 (fwd: GCCTT CCCTTACACCAATAGAG, rev: GTTGGTGCAGTGGAGTATGT), SCNN1A (fwd: GGCTGTGCCTACATCTTCTATC, rev: GAGAAGTCAACCTGGAGC TTATAG), STAT5A (fwd: GCCACCATCACGGACATTAT, rev: CAAACTT GGTCTGGGTCTTCA), IGFBP5 (fwd: GAAGAAGGACCGCAGAAAGAA, rev: CTCAGACTCCTGTCTCATCTCA), TNFRSF21 (fwd: CCAACTCTTC TGCCTCTGTTAG, rev: GAGGGTCTTGTTCACGTCTTC), GDA (fwd: CGCACACTGTCCCAATTCTA, rev: TCTGTACCCAGCCCTATCTT). Primers for ChIP-qPCR were as follows in 5'-3' orientation: GATA3 promoter (fwd:TTGGTGCTCGCGATTGAA, rev: AAATGCTGACTTCTGAGGCTAA), GATA3 enhancer (fwd: ATCCATCAGCCCTTCTTTCTG, rev: GCGCCAT CTACTGGGTTATT).

Reporting summary

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

Data availability

Data generated in this study: RNA-seq data of TNBC cell lines treated with EZH2i and AKTi have been deposited at the Gene Expression Omnibus for MDA-MB-468 (GSE205729), SUM149PT (GSE205730), and HCC38, HCC1937 and HCC1395 (GSE251708). RNA-seq data for MDA-MB-468 cells treated with docetaxel or EZH2/AKTi have been deposited under GSE250632. CUT&RUN data are available under PRJNA1054805 and ATAC-seq data have been deposited under GSE252382. CyCIF images of PDX004 tumours are available online (https://s3.amazonaws.com/ www.cycif.org/cichowski-santagata-sorger-2024/index.html). Publicly available cancer datasets: to determine EZH2 mRNA levels in TNBC tumours and matched normal breast tissue, data were extracted from the TCGA Firehose Legacy dataset⁸⁰. TNBC tumours were identified from the dataset if they were negative for ER, PR and HER2 in the clinical annotation. RNA-seq read counts were collected from the CCLE DepMap portal (DepMap Public 21Q4). For machine learning, RNA-seq raw counts and corresponding clinical data were also obtained from of BRCA TNBC samples in the TCGA using TCGAbiolinks package in R⁸⁰. Mutation and copy-number variation and the mRNA levels for each TCGA tumour were collected from the Firehose Legacy dataset.

Hi-C data from MCF10A (ENCFF977XWK) and MCF7 (ENCFF420JTA) cells were extracted from ENCODE and visualized in the IGV. FOXO1 ChIP-seq data were extracted from ENCODE (ENCSR3210AA) and visualized in the IGV.

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Author contributions A.E.S. and K.C. conceived the study. A.E.S., K.C., N.P., YY, V.T.M.N., J.-R.L., P.K.S., K.H., S.S. and S.R.V.K. designed the study. A.E.S., N.P., YY, C.L.R., A.K., A.G., P.L., Y.X., V.T.M.N., G.M.M., N.F.P., M.W., K.O., R.A.D., D.X., J.J.Z. and J.-R.L. acquired the data and/or generated key resources. A.E.S., K.C., N.P., YY, C.L.R., A.K., Y.X., K.M., K.O., J-R.L., P.K.S., K.H., S.S. and S.R.V.K. analysed the data. A.E.S., K.C., N.P., YY, Y.X., K.D., S.M., A.C.G.-C., S.M.T, Z.L., D.A.B., P.K.S., K.H., S.S. and S.R.V.K. interpreted the data. A.E.S., K.C., YY. and S.R.V.K. drafted and edited the manuscript.

Competing interests K.C. is an advisor at Genentech and serves on the scientific advisory board of Erasca. S.M.T. has consulting or advisory roles at Novartis, Pfizer, Merck, Lilly, Nektar, NanoString Technologies, AstraZeneca, Puma Biotechnology, Genentech/Roche, Eisai, Sanofi, Bristol Myers Squibb, Seattle Genetics, Odonate Therapeutics, OncoPep, Kyowa Hakko Kirin, Samsung Bioepis, CytomX Therapeutics, Daiichi Sankyo, Athenex, Gilead, Mersana, Certara, Chugai Pharma, Ellipses Pharma, Infinity, 4D Pharma, OncoSec Medical, BeyondSpring Pharmaceuticals, OncXerna, Zymeworks, Zentalis, Blueprint Medicines, Reveal Genomics and ARC Therapeutics; and received institutional research funding from Genentech/Roche, Merck, Exelixis, Pfizer, Lilly, Novartis, Bristol Myers Squibb, Eisai, AstraZeneca, NanoString Technologies, Cyclacel, Nektar, Gilead, Odonate Therapeutics, Sanofi and Seattle Genetics. P.K.S. is a co-founder and member of the BOD of Glencoe Software, member of the BOD for Applied Biomath, member of the scientific advisory board for RareCyte, NanoString and Montai Health, holds equity in Glencoe, Applied Biomath and RareCyte, consults for Merck, and has received research funding to the institution from Novartis and Merck in the past 5 years. A.C.G.-C. has grant/research funding to institution from Gilead Sciences, AstraZeneca, Daiichi-Sankyo, Merck, Zenith Epigenetics, Bristol-Myers Squibb, Novartis, Foundation Medicine and Biovica; serves as a consultant and member of the scientific advisory board for AstraZeneca, Daiichi-Sankyo; and has other financial or materials support from Roche/Genentech, Gilead Sciences, AstraZeneca, Novartis and Merck. K.H. is a consultant for and a co-founder of Dania Therapeutics Aps and a scientific advisor for Hannibal Health Innovation. S.R.V.K. is a founder and consultant at Faeth Therapeutics and Transomic Technologies. D.A.B. is a consultant for N of One/QIAGEN and Tango Therapeutics; is a founder and shareholder in Xsphera Biosciences; has received honoraria from Merck, H3 Biomedicine/Esai, EMD Serono, Gilead Sciences, Abbvie and Madalon Consulting; and has received research grants from BMS, Takeda, Novartis, Gilead and Lilly. The other authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Karen Cichowski. Peer review information *Nature* thanks Charles Sawyers and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Reprints and permissions information is available at http://www.nature.com/reprints.



Extended Data Fig. 1 | **Extended data in relation to Fig. 1a-e. a**, Related to Fig. 1a. EZH2 mRNA levels within normal tissue (*n* = 111), or TNBC tumours resected at stage I (*n* = 19), stage II (*n* = 74), stage III (*n* = 19), or stage IV (*n* = 2). Box shows 10–90 percentiles with line at median. **b**, Treatment schema for all in vitro studies. **c-d**, Immunoblot depicting relative levels of AKT target inhibition (pPRAS40) and EZH2 target inhibition (H3K27me3) and relevant loading controls in sensitive (c) and resistant (d) cell lines from panel in Fig. 1c.

Experiment was repeated at least 3 times. **e**, Dose dependence of cell death induced by the combination of EZH2i (tazemetostat) and AKTi (ipatasertib) measured by Incucyte Live Cell Imaging. Data for 5 μ MAKTi are presented as the 72 h time point in Fig. 1d. n = 8. p*** <0.001 measured by unpaired one-tailed heteroscedastic Student's T-test. Data are images from 8 independent wells. **f**, Synergy plots using Gaddum's non-interaction model (HSA) for cells treated with AKTi (ipatasertib) and/or EZH2i (tazemetostat).



Extended Data Fig. 2 | See next page for caption.

Extended Data Fig. 2 | Extended data in relation to Fig. 1. a, Relative change in cell number after 4 days of treatment with vehicle, AKTi (MK2206) and/or EZH2i (tazemetostat) (Day 4 versus Day 0). b, Relative change in TNBC cell numbers after 4 days of treatment with vehicle, AKTi (ipatasertib), EZH2i (tazemetostat), and/or EEDi (MAK683) (Day 4 versus Day 0). Immunoblot (repeated at least 3 times) depicting relative levels of AKT target inhibition (pPRAS40) and EZH2 target inhibition (H3K27me3) and relevant loading controls. c, Relative change in cell numbers after 4 days of treatment with vehicle, AKTi (ipatasertib) and/or EZH2i (tazemetostat) (Day 4 versus Day 0) in indicated cell lines. d, Immunoblot depicting relative levels of AKT target inhibition (pPRAS40) and EZH2 target inhibition (H3K27me3) and relevant loading controls in indicated cell lines from panel in (c). e, Crystal violet staining of SUM149PT cells treated with vehicle, AKTi (ipatasertib) and/or EZH2i (tazemetostat) and fixed after 10, 20, or 30 days of treatment. **f**, Relative change in cell number of MDA-MB-468 cells after treatment. Left – pre-treatment with EZH2i for 5 days followed by 4 days of combined treatment of EZH2i and/or AKTi. Middle – pre-treatment with EZH2i for 5 days followed by switch to vehicle or AKTi for an additional 4 days. Right – pre-treatment with EZH2i for 5 days followed by drug holiday for 4 days, followed by vehicle or AKTi treatment for an additional 4 days. Immunoblot on right depicts relative levels of AKT target inhibition (pPRAS40) and EZH2 target inhibition (H3K27me3) and relevant loading controls. For all subfigures, n = 3, data are mean ± s.d. of biological independent samples and p values were measured by unpaired one-tailed heteroscedastic Student's T-test.



Extended Data Fig. 3 | See next page for caption.

Extended Data Fig. 3 | **Extended data in relation to Fig. 1f-j. a**, EZH2 mRNA expression levels in sensitive and resistant TNBC cell lines from CCLE RNAseq dataset. Sensitive cells are ranked in order of sensitivity measured by amount of cell death observed in Fig. 1c. **b**, Immunoblot depicting PTEN expression and *PIK3CA* mutational status of TNBC cell lines with Fisher's exact test panel. Experiment was completed at least 3 times with similar results. **c**, Graph depicting the relative tumour volume of MDA-MB-468 orthotopic xenografts over time treated with Vehicle, AKTi (ipatasertib) and/or EZH2i (tazemetostat). Note that established tumours (100mm³) were first pretreated with vehicle or tazemetostat for 7 days prior to the addition of these agents. p value determined using mixed effects models (REML) ANOVA between AKTi and Combo. *n* = 10–13 tumours per arm. Data are mean ± s.e. of biological independent samples.

d, Kaplan-Meier survival plot of mice bearing MDA-MB-468 orthotopic xenografts treated with vehicle or EZH2i+AKTi. Treatment ended after day 28 and animals were monitored twice weekly for humane endpoint. Significance measured by Mantel-Cox Log Rank test. **e**, Graph depicting percent change in body weight of tumour bearing mice treated with the indicated drugs during the treatment duration. Data are mean \pm s.e. of biological independent samples. n = 6 or 7 mice per condition. **g**, Copy number plots of chromosomes bearing Akt3 (e), Met (f), and Ezh2 (g) isolated from untreated GEMM organoid allograft tumours from K8-CreER; Trp53^{nV/n} mice implanted orthotopically into nude mice. **h**, Photographs of H&E-stained (left) or EZH2 immunohistochemistry (right) sections of a normal mouse mammary fat pad or tumour from Fig. 1h. Scale bar = 150 µm.



Extended Data Fig. 4 | **Extended data in relation to Fig. 11.** Photographs of H&E-stained (top) or Ki67 immunohistochemistry (bottom) tumour sections at the end of study depicted in 11. F = fibrosis; N = necrosis; C = calcification. Scale bar = 150 μ m.



Extended Data Fig. 5 | See next page for caption.

Extended Data Fig. 5 | Extended data in relation to Fig. 2. a, differentially expressed genes between EZH2i+AKTi and DMSO treated SUM149PT cells. A subset of luminal and basal breast markers are highlighted. Padj is DESeq2 p value adjusted for multiple hypothesis testing. b, ssGSEA z scores of LIM_ MAMMARY_LUMINAL_MATURE gene signatures in MDA-MB-468 cells. c, Transcriptional heatmap of significantly differentially expressed genes in gene signatures from (Fig. 2a) in MDA-MB-468 cells. d, Correlation coefficient of cell lines treated indicated drugs with basal TNBCtypes. e-f, RNA-seq normalized counts of ER (e) and AR (f) in SUM149PT and MDA-MB-468 cells. g, GATA3 protein expression in two sensitive cell lines treated with indicated drugs. h, Immunoblot of SUM149PT cells transfected with siControl or siGATA3 and treated with indicated drugs. i, Synergy scores using Gaddum's non-interaction model (HSA) in cells stably transduced with shRNA or sgRNA against GATA3 or a control sequence. Cells were treated with various concentrations of AKTi and/or EZH2i. **j**, Synergy plots using Gaddum's non-interaction model (HSA) for cells stably transduced with shRNA against GATA3 or a control sequence and then treated with indicated drugs. **k**, Relative change in cell number of MDA-MB-468 stably transduced with sgRNAs or transfected with siRNAs against a control sequence or GATA3 and then treated with indicated drugs. **l**, immunoblot of p27 and loading control of MDA-MB-468 cells treated with indicated drugs for 16 h. **m**, ssGSEA z scores of Hallmarks – E2F target gene signature in SUM149PT and MDA-MB-468 cells. **n**, immunoblot of p27 and loading control GAPDH of protein lysates from MDA-MB-468 cells treated with indicated drugs, in the presence of siRNAs against a control sequence or GATA3. For all subfigures, data are mean ± s.d. of biological independent samples (*n* = 3), immunoblots were repeated at least 3 times, box plots show range of data with line at mean, AKTi = ipatasertib, and EZH2i = tazemetostat.



Extended Data Fig. 6 | See next page for caption.

Extended Data Fig. 6 | Data related to Fig. 3. a, gene set enrichment analysis of open peaks enriched in sensitive and resistant TNBC cell lines at baseline as assessed by ATAC-seq. Adjusted p values are FDR calculated using the Benjamini Hochberg method within the GSEA software. **b**-**c**, leading edge plot of gene set enrichment analysis of CHARAFE BREAST CANCER BASAL VS MESENCHYMAL DOWN (mesenchymal genes, (b)) and CHARAFE BREAST CANCER BASAL VS MESENCHYMAL UP (basal genes, (c)) in sensitive and resistant cell lines at baseline as measured by RNA-seq. Adjusted p values are FDR calculated using the Benjamini Hochberg method within the GSEA software. **d**, heatmap of correlation coefficients of differentially expressed genes in TNBC cell lines generated in this study as DMSO treated samples or sourced from the CCLE dataset. **e**, relative cell counts of five additional TNBC cell lines predicted to be sensitive or resistant by machine learning models. Data are mean \pm s.d. of biological independent samples. n = 3. **f**, performance metrics for machine learning models. **g**, oncoprint of TNBC tumours from TCGA firehose dataset with alterations for AKT3, PTEN, INPP4B, and PIK3CA denoted.



Extended Data Fig. 7 | **Data related to Fig. 4. a**, Chromatin looping architecture of GATA3 enhancer linked to GATA3 promoter using MCF10A HiC data from ENCODE. **b**, Genome browser view of GATA3 enhancer with ATAC-seq and CUT&RUN for H3K27me3 data from MDA-MB-468 cells treated with EZH2i

and/or AKTi. Data is overlaid with HiC data for MCF10A and MCF7 cells. Note the ATAC-seq and CUT&RUN data are identical to in Fig. 4a for direct comparison purposes. **c**, schematic for siRNA screen completed in Fig. 4b.



Extended Data Fig. 8 | **Data related to Fig. 4.** a, immunoblot of FOXO1 and pFOXO1 expression in TNBC cell lines treated with EZH2i and/or AKTi. Experiment was repeated at least 3 times. **b**, RT-qPCR of FOXO1 expression after EZH2i and/or AKTi treatment in three sensitive TNBC cell lines. Values indicate z scores of three independent biological samples. **c**, genome browser view of FOXO1 locus with CUT&RUN data using H3K27me3 and H3K4me3 antibodies in chromatin from MDA-MB-468 cells treated with EZH2i and/or AKTi. **d**, genome browser view of GATA3 promoter with FOXO1 ChIP-seq occupancy (accessed from ENCODE). **e**, genome browser view of GATA3 enhancer with FOXO1 ChIP-seq occupancy (accessed from ENCODE).



 $\label{eq:constraint} Extended \, Data Fig. \, 9 \, | \, \text{See next page for caption}.$

Extended Data Fig. 9 | Data related to Fig. 5. a, ssGSEA z scores of HALLMARK_ APOPTOSIS gene signatures in SUM149PT and MDA-MB-468 cells treated with AKTi and/or EZH2i. b, Relative BMF mRNA expression (RT-qPCR) in SUM149PT cells treated with EZH2i and/or AKTi. c, Protein expression of BMF in HCC1395 cells treated with EZH2i and/or AKTi with stable HA knocked-into the endogenous BMF locus. d-e, Relative change in cell number (f) or BMF expression (e) of SUM149PT cells transfected with siControl or siBMF e and then treated with EZH2i and/or AKTi. f, Transcriptional heatmap depicting expression of significantly differentially expressed genes from Fig. 5d in MDA-MB-468 cells. g, involution gene mRNA expression measured by RT-qPCR in SUM149PT cells transfected with siControl or siSTAT3 in response to indicated drugs. h, Relative change in cell number of MDA-MB-468 cells treated with EZH2i+AKTi or docetaxel (10 nM) after 4 days. i, BMF expression (z-score of DESeq2 normalized counts) in MDA-MB-468 cells treated with EZH2i+AKTi or docetaxel. **j**, Immunoblot depicting activation of STAT3 (pSTAT3) and target inhibition after treatment with EZH2i+AKTi or docetaxel in MDA-MB-468 cells. **k**-I, ssGSEA z scores (k) or heatmap (l) of Mammary Gland Involution gene signatures in MDA-MB-468 cells treated with indicated drugs. **m**,**n**, Immunoblots depicting confirmation of STAT3 knockdown and inhibition of AKT and EZH2 targets in SUM149PT (m) or MDA-MB-468 (n) cells. Related to Fig. 5i. **o**, immunoblot of SUM149PT cells transfected with siControl or siJAK1 and then treated with indicated drugs. Related to Fig. 5j. For all subfigures, data are mean \pm s.d. of biological independent samples (n = 3), p values measured by unpaired one-tailed heteroscedastic Student's T-test, box plots show range of data with line at mean, immunoblots were repeated at least 3 times, EZH2i = tazemetostat, AKTi = ipatasertib, and docetaxel dosed at 10 nM.



Extended Data Fig. 10 | See next page for caption.

Extended Data Fig. 10 | Data related to Fig. 6. a, relative IL6 protein (z score) in SUM149PT and MDA-MB-468 cells after 24 h of treatment with EZH2i (tazemetostat) and/or AKTi (ipatasertib). **b**, Relative IL6R expression in SUM149PT and MDA-MB-468 cells transfected with siRNA against a control sequence or IL6R and then treated with vehicle or EZH2i+AKTi. Related to Fig. 6a. **c**, Relative cell numbers of SUM149PT and MDA-MB-468 cells treated with vehicle, EZH2i+AKTi, and/or neutralizing antibody against IL6R (tocilizumab). **d**, relative IL6 protein level (ELISA) in MDA-MB-468 cells transfected with siControl or siSTING and treated with indicated drugs for 24 hr. *n* = 2. **e**, 2'3'-cGAMP levels measured by ELISA in MDA-MB-468 cells after treatment with indicated drugs. **f**, volcano plot depicting-log10(FDR) versus

log2(fold change) for expression of endogenous retroviruses in EZH2i vs DMSO treated MDA-MB-468 cell line. **g**, transcriptional heatmap of BMF levels in a panel of TNBC cell lines treated with EZH2i and/or AKTi and harvested after 24 h. BMF levels were normalized to STAU1 and then relative expression was normalized to each cell line's DMSO samples. Data for sensitive cell lines is generated from same experiment as Fig. 5a. Heatmap values indicate fold change. **h**, relative cell numbers of SUM159PT cells transduced with GFP or GATA3 overexpression construct treated with EZH2i+AKTi and/or STING agonist ADUS100. For all subfigures, data are mean \pm s.d. of biological independent samples. Unless indicated, all experiments are n = 3. p values measured by unpaired one-tailed heteroscedastic Student's T-test.

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Corresponding author(s): Karen Cichowski

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		Our web collection on <u>statistics for biologists</u> contains articles on many of the points above.

Software and code

Policy information about availability of computer code

No software was generated in this study. Incucyte Live Cell Imaging software was used for image acquisition and data analysis. BioRad Data collection Chemidoc was used for acquisition of some western blot images. For CyCIF, automatic imaging was conducted using a RareCyte Cytefinder II HT and acquired images were processed using the Docker-based NextFlow pipeline MCMICRO. Statistical tests including unpaired t-test, ANOVA, and Mann-Whitney tests were conducted using Graphpad Prism version 10. RStudio version Data analysis 2023.06.0+421 was used. Integrated Genome Viewer version 2.16.1 was used. RNAseq. Raw data was mapped to the Hg19 (SUM149PT, MDA-MB-468) or hg38 MDA-MB-468 docetaxel experiment, HCC38, HCC1395, HCC1937) genome using STAR and count files were made using HTSeq. DESeq2 was used to normalize counts (mean-ratio method), calculate total reads, and determine differentially expressed genes. Volcano plots were generated using EnhancedVolcano and heatmaps were generated using pheatmap. DESeq2 was used to determine differentially expressed genes for public RNAseq raw counts collected from CCLE database. Volcano plots were generated using ggplot2 package in R and heatmaps were generated using ComplexHeatmap package in R. ATAC-seq. ATAC-seq data was analyzed using ENCODE pipeline (https://www.encodeproject.org/atac-seq/) and aligned to hg38 reference. MACS2 was used to identify peaks indicating open chromatin region. We filtered out non-reproducible peaks by selecting peaks that were detected in replicates of each condition for each cell line, then peaks were merged for overlapping region across all conditions and samples using GenomicRanges package in R. The consensus peaks were annotated using ChIPseeker package in R. Using featureCounts in Rsubread package in R, we counted all read over the consensus peak regions, followed by differential accessible peak analysis using DESeq2. Motif discovery and analysis was performed using RGT-HINT. TF binding sites were identified with JASPAR motifs. TF activity scores were estimated based on the TF footprint profiles. The ranked genes were used for gene set enrichment analysis with fgsea package in R. Gene set were collected from entire MSigDB using msigdbr package in R.

Copy Number Analysis of TP53 -/- allograft tumors was completed by analyzing low pass whole genome sequencing data using the CNVkit pipeline to process the germline sequencing raw data. The scatter plot was generated using CNVkit library in Python.

ssGSEA and GSEA. Single sample gene set enrichment analysis was performed using GenePattern (https://www.genepattern.org/). Gene set enrichment analysis was performed using software available at http://www.gsea-msigdb.org/.

TNBCtype. Molecular subtyping of samples using RNAseq values was performed using the TNBCtype tool.

CUT&RUN. Paired-end fastq files were aligned to hg38 reference genome using Bowtie2 with setting "—very-sensitive –no-mixed –nodiscordant –phred33 -I 10 -X 700". Sequencing reads were also aligned to Ecoli genome to map spike-in reads. For spike-in normalisation, the total number of mapped reads to Ecoli genome was used to calculate normalisation factor for CUT& RUN samples. Sam files were converted to bam files using samtools. Bigwig files were generated from bam files using Deeptools. Genome browser tracks of big file files were generated using IGV. Peaks were called using MACS2 using callpeak function with "-f BAME -keep-dup 1 -q 0.05" and IgG was used as controls. DeSeq2 analysis from DiffBind R package with option "spikein=True" was used to compare differential binding between conditions (FDR < 0.1).

ERV expression. In order to count reads for ERVs, ERV loci information was collected from the ERVmap database. Salmon (v0.14.1) was used to create a customized reference transcriptome set which includes human genome transcriptome (hg38) and ERVmap database. DESeq2 was used for counts normalization and differential expression analysis.

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Data generated in this study: RNAseq data of TNBC cell lines treated with EZH2i and AKTi are deposited on GEO for MDA-MB-468 (GSE205729), SUM149PT (GSE205730), and HCC38, HCC1937, HCC1395 (GSE251708). RNAseq data for MDA-MB-468 cells treated with docetaxel or EZH2i+AKTi is deposited under GSE250632. CUT&RUN data is under PRINA1054805 and ATAC-seq data is deposited under GSE252382. CyCIF images of PDX004 tumors can be found at this link: https://s3.amazonaws.com/www.cycif.org/cichowski-santagata-sorger-2024/index.html.Publicly available cancer datasets: To determine EZH2 mRNA levels in TNBC tumors and matched normal breast tissue, data was extracted from the TCGA Firehose Legacy dataset56. TNBC tumors were identified from the dataset if they were negative for ER, PR, and HER2 in the clinical annotation. RNA seq read counts were collected from the CCLE DepMap portal (DepMap Public 21Q4). For machine learning, RNAseq raw counts and corresponding clinical data were also obtained from 50 BRCA TNBC samples in TCGA using TCGAbiolinks package in R57. Mutation and copy number variation, mRNA level for each TCGA tumor were collected from Firehose Legacy dataset. Hi-C data from MCF10A (ENCFF977XWK) and MCF7 (ENCFF420ITA) cells were extracted from ENCODE and visualized in the Integrative Genome Viewer (IGV). FOXO1 ChIPseq data was extracted from ENCODE (ENCSR3210AA) and visualized in IGV.

Human research participants

Policy information about studies involving human research participants and Sex and Gender in Research.

Reporting on sex and gender	No human research participants were recruited in this study.
Population characteristics	See above.
Recruitment	No participants were recruited.
Ethics oversight	No study protocol was created for this.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

X Life sciences

Behavioural & social sciences

Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see <u>nature.com/documents/nr-reporting-summary-flat.pdf</u>

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.	
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Sample size	For in vitro experiments, sample size > or = 3 and was not predetermined by a statistical method. Experiments were completed at least 3 times. For in vivo experiments, sample size was calculated that if we used 10 mice per treatment group, we would have yield 87% power (2 sample t test, alfa of 0.05, SD 0.72) to detect a 50% reduction compared to the initial tumor volume.
Data exclusions	For Figure 4f, outliers from 10 technical replicates were excluded using the IQR method (< Q1 - (1.5 * IQR); > Q3 + (1.5 * IQR). No other data was excluded.
Replication	Experiments were completed at least three times. For all in vitro experiments, there were at least 3 biologically independent samples per experimental condition, with the exception of Extended Data Figure 10D which had n = 2.
Randomization	For in vivo experiments, animals were randomized into treatment arms. Animals were enrolled as cohorts when their tumors reached ~100-300 mm3 volume and randomized into treatment arms. For in vitro experiments, samples were not randomized but were seeded from the same cell solution across all relevant conditions to control for seeding density/population.
Blinding	Blinding was not possible as animals needed to be clearly identified for each treatment arm. Analysis for incucyte data was unbiased as 4-5 images were used for training the analysis definition which was then uniformly applied to all images (8 images per time point per condition per well). No other experiments were blinded.

Reporting for specific materials, systems and methods

Methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials	&	experimental	S	vstems
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	1 /		
n/a	Involved in the study	n/a	Involved in the study
	X Antibodies	\ge	ChIP-seq
	Eukaryotic cell lines	\boxtimes	Flow cytometry
\boxtimes	Palaeontology and archaeology	\boxtimes	MRI-based neuroimaging
	Animals and other organisms		
\boxtimes	Clinical data		
\boxtimes	Dual use research of concern		

Antibodies

Antibodies used	For immunoblot: H3K27me3 (dilution 1:1000, 9733S, Cell Signaling Technologies, AB_2616029); H3 (4dilution 1:1000,499S, Cell Signaling Technologies, AB_10544537); PRAS40 (dilution 1:1000, 2610S, Cell Signaling Technologies, AB_916206); pPRAS40 (dilution 1:1000, 2997S, Cell Signaling Technologies, AB_2258110); GAPDH (dilution 1:1000, 2118S, Cell Signaling Technologies, AB_561053); GATA3 (1:250 dilution, 558686. BD BioSciences, AB_2108590); pSTAT3 (dilution 1:1000, 3332S, Cell Signaling Technologies, AB_628292); STAT3 (dilution 1:1000, 9139, Cell Signaling Technologies, AB_331757); JAK1 (dilution 1:1000, 3332S, Cell Signaling Technologies, AB_2128499), p27 (dilution 1:1000, sc-528, Santa Cruz, AB_632129), FOXO1 (dilution 1:1000, 9454, Cell Signaling Technologies, AB_823503), FOXO1-pS265 (dilution 1:500, 9461, Cell Signaling Technologies, AB_329831), TBK1 (dilution 1:1000, 3504S, Cell Signaling Technologies, AB_2255663), TBK1-pS172 (dilution 1:1000, 5483S, Cell Signaling Technologies, AB_10 6 93472), STING (dilution 1:1000, 13647S, Cell Signaling Technologies, AB_2732796).			
	Anti-IL6R antibody is tocilizumab and was supplemented at 50 g/mL (Catalog #A2012-5MG, Selleckchem). For IHC: CK14 (Abcam # ab77684, clone LL002, conjugated to FITC, 1:500 dilution), CK8 (eBioscience, # 11-9938-80, clone LP3K, conjugated to FITC, 1:400 dilution), panCK (eBioscience, # 53-9003-82, clone AE1/AE3, conjugated to AF488, 1:1000 dilution), EZH2 (CST, #45638, clone D2C9, conjugated to AF647, 1:100 dilution), cPARP (CST, #6987S, clone D64E10, conjugated to AF647, 1:100 dilution), E-Cadherin (CST, #9835S, clone 24E10, conjugated to AF647, dilution 1:600), anti-Rabbit IgG (Invitrogen # A-31572, conjugated to AF555, 1:1000 dilution), and anti-Mouse IgG (Invitrogen, #A-31571, conjugated to AF647, 1:1000 dilution).			
Validation	GATA3 antibodies were validated for western blot by detecting loss of signal with siRNA, shRNA, and sgRNA mediated knockdown of the relevant target. STAT3 and pSTAT3 antibodies were validated for western blot by loss of detection after siRNA mediated knockdown of JAK1. TBK1 antibody was validated for western blot by loss of detection after siRNA mediated knockdown of JAK1. TBK1 antibody was validated for western blot by loss of detection after siRNA mediated knockdown of JAK1. TBK1 antibody was validated for western blot by loss of detection after siRNA mediated knockdown of JAK1. TBK1 antibody was validated for western blot by loss of detection after siRNA mediated knockdown of TBK1. STING antibody was validated for western blot by loss of detection after siRNA mediated knockdown of STING. H3K27me3 antibody was validated for western blot after detecting loss of signal after treatment with EZH2 inhibitor. pRAS40 antibody was validated for western blot after detecting loss of signal after treatment with AKT inhibitor. GAPDH, PRAS40, and H3 antibodies are standard loading control antibodies that are well established by the vendors and many citations supporting their specificity and were not further validated in			

Eukaryotic cell lines

Policy information about <u>cell lines</u>	s and Sex and Gender in Research
Cell line source(s)	MDA-MB-436, HCC1395, HCC1937, BT20, 293T, IMR90, BJ, HCC70, HCC1187, HC2157, DU4457 and MDA-MB-157 cell lines were purchased from ATCC. SUM149PT and SUM159PT cell lines were a gift from Frank McCormick (University of California San Francisco). MDA-MB-468, HCC38, HCC143, and HCC1806 cell lines were a gift from Alex Toker (Beth Israel Deaconess Medical Center). MDA-MB-231 and BT549 cell lines were a gift from William Hahn (Dana-Farber Cancer Institute). RPE1 cells were purchased from Clontech. MCF10A cells were a gift from Jayanta Debnath (University of California San Francisco).
Authentication	All cell lines were authenticated using STR DNA profiling.
Mycoplasma contamination	Cells were routinely found to have no Mycoplasma contamination using Lonza MycoAlert PLUS Mycoplasma Detection Kit.
Commonly misidentified lines (See <u>ICLAC</u> register)	No commonly misidentified lines were used in this study.

Animals and other research organisms

Policy information about studies involving animals; <u>ARRIVE guidelines</u> recommended for reporting animal research, and <u>Sex and Gender in</u> <u>Research</u>

Laboratory animals	SUM149PT, MDA-MB-468 and GEMM allograft xenografts were completed in nu/nu mice from Charles River Laboratories. Patient derived xenografts HCI-025 and HCI-004 were completed in NSG mice from Jackson Laboratory. Trp53fl/fl mice were used to generate p53-/- organoids which were transplanted orthotoically into nude mice. Trp53L (B6.129P2-Trp53tn1Brn/J, Stock No: 008462), and R26Y (B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J, Stock No: 006148) mice were obtained from The Jackson Laboratory and crossed to generate Trp53L/L;R26Y mice. Ad-K8-Cre was introduced into ducts of the #4 inguinal mammary gland via intraductal injection at approximate 2-3 months of age and monitored twice weekly until tumors formed. Temperature in rooms housing mice were kept stably between 68-75C. Humidity range of rooms housing mice was kept stably 35-65%, with 50% humidity being considered optimal. Animals experienced 12 hours of light during the day and 12 hours of dark during the night with light intensiy between 100-250 lux. Mice were offered diet ab libitum in food hoppers hung from the top of the cage with Pico 5052 Irradiated Rodent Chow or Pico 5058 Irradiated Mouse Chow. Mouse were offered water ab libitum. Mice were housed in Allentown ventilated cages. Mouse cages were filled with bedding approximately 1/2 inch in depth with Alpha-blend or crinkled paper (nude mice received paper-based bedding only). Mice were offered gnawing enrichment in the form of nestlets or popsicle sticks (nude mice only offered popsicle sticks).
Wild animals	No wild animals were used in this study.
Reporting on sex	All xenografts were completed in female nulliparous mice.
Field-collected samples	No field-collected samples were used in this study.
Ethics oversight	All mouse work was done in compliance with the Institutional Animal Care and Use Committee (IACUC) at Brigham and Women's Hospital.

Note that full information on the approval of the study protocol must also be provided in the manuscript.