ZC3H18 specifically binds and activates the \textit{BRCA1} promoter to facilitate homologous recombination in ovarian cancer

Arun Kanakkanthara\textsuperscript{1,2}, Catherine J. Huntoon\textsuperscript{1}, Xiaonan Hou\textsuperscript{3}, Minzhi Zhang\textsuperscript{3}, Ethan P. Heinzen\textsuperscript{4}, Daniel R. O’Brien\textsuperscript{4}, Ann L. Oberg\textsuperscript{4}, S. John Weroha\textsuperscript{3}, Scott H. Kaufmann\textsuperscript{1,2} & Larry M. Karnitz\textsuperscript{1,2*}

Reduced BRCA1 expression causes homologous recombination (HR) repair defects in high-grade serous ovarian cancers (HGSOCs). Here, we demonstrate that \textit{BRCA1} is transcriptionally activated by a previously unknown function of ZC3H18. We show that ZC3H18 is a DNA-binding protein that interacts with an E2F site in the \textit{BRCA1} promoter where it facilitates recruitment of E2F4 to an adjacent E2F site to promote \textit{BRCA1} transcription. Consistent with ZC3H18 role in activating \textit{BRCA1} expression, ZC3H18 depletion induces \textit{BRCA1} promoter methylation, reduces \textit{BRCA1} expression, disrupts HR, and sensitizes cells to DNA crosslinkers and poly(ADP-ribose) polymerase inhibitors. Moreover, in patient-derived xenografts and primary HGSOC tumors, ZC3H18 and \textit{E2F4} mRNA levels are positively correlated with \textit{BRCA1} mRNA levels, further supporting ZC3H18 role in regulating \textit{BRCA1}. Given that ZC3H18 lies within 16q24.2, a region with frequent copy number loss in HGSOC, these findings suggest that ZC3H18 copy number losses could contribute to HR defects in HGSOC.
Homologous recombination (HR) is a high-fidelity DNA repair mechanism that requires the sequential activities of a series of proteins, including BRCA1 and BRCA2 tumor suppressors. Defects in HR are a defining feature of high-grade serous ovarian cancers (HGSOCs), the most common and lethal ovarian cancer subtype. The most frequent causes of HR defects are deleterious mutations in BRCA1 and BRCA2 (ref.1), which are associated with increased response rates to platinum-based therapies, enhanced disease-free survival, and improved overall survival. HGSOCs with deleterious BRCA1/2 mutations are also sensitive to poly(ADP-ribose) polymerase (PARP) inhibitors.

Notably, many HGSOCs have HR defects despite a lack of mutations in BRCA1/2 and other known DNA repair genes. A substantial fraction of these are due to reduced BRCA1 transcription, which is associated with HR defects in HGSOCs. Two known mechanisms that cause reduced BRCA1 expression include (1) hypermethylation of the BRCA1 promoter, which occurs in 8–15% of HGSOCs, and (2) mutational inactivation of CDK12 (ref.11), an RNA polymerase II C-terminal domain (CTD) kinase that regulates the transcription of BRCA1 and other genes. Additionally, BRCA1 transcription is controlled by a complex array of transcription factors, coactivators, and corepressors that interact with the BRCA1 promoter. However, a complete understanding of the transcriptional regulation of BRCA1 is lacking.

Here, we report on a previously uncharacterized mode of BRCA1 transcriptional regulation. We show that BRCA1 transcription is regulated by ZC3H18, which we demonstrate has a previously unknown biochemical function: ZC3H18 is a DNA-binding protein that interacts with an E2F site in the BRCA1 promoter and that activates transcription. Accordingly, these studies expand the known roles for ZC3H18, which was previously shown to participate in RNA processing by mediating mRNA export, degradation, and transcription of a subset of protein-coding genes through its association with the mRNA cap-binding complex and the nuclear exosome-targeting complex. This study also shows that ZC3H18 binding to an E2F site in the BRCA1 promoter enhances the association of E2F4 with an adjacent E2F site to activate BRCA1 transcription. Consistent with these observations, ZC3H18 and E2F4 mRNA levels correlated with BRCA1 mRNA levels in primary human HGSOC tumors and patient-derived xenograft (PDX) models. Collectively, these results discover an additional biochemical function for ZC3H18: uncover a uncharacterized mechanism of BRCA1 transcriptional regulation; and because ZC3H18 is located in a region (chromosome 16q24.2) of recurrent copy number loss in HGSOC, suggest that reduced ZC3H18 levels may be an unrecognized contributor to diminished BRCA1 expression and HR defects in HGSOC.

Results

ZC3H18 depletion induces an HR defect and DNA damage sensitivity. Copy number losses in chromosomal region 16q24.2 are a common event in HGSOC (Supplementary Fig. 1a). Indeed, some studies have reported 16q24.2 loss to be among the most frequent copy number variation in HGSOC, raising the possibility that genes located within this region could impact HR. To assess the potential role of genes in this region in HR, we conducted an siRNA screen of known protein-coding genes at 16q24.2, focusing the present studies on BRCA1. Consistent with the RNA-seq analysis, ZC3H18 depletion profoundly decreased BRCA1 mRNA (Fig. 2b) and protein levels (Fig. 2a) in multiple ovarian cancer cell lines and in xenografted OVCAR-8 cells (Supplementary Fig. 2c). Moreover, expression of siRNA-resistant ZC3H18 restored BRCA1 mRNA (Fig. 2c) and protein levels (Supplementary Fig. 4b) in ZC3H18 siRNA-transfected cells confirming that ZC3H18 facilitates accumulation of BRCA1 mRNA and protein. Finally, because multiple HR-associated genes were downregulated by ZC3H18 depletion (Supplementary Data 1 and Supplementary Fig. 3), we next asked whether the loss of BRCA1 was a major contributor to the HR defect caused by ZC3H18 depletion. As shown in Fig. 2d, heterologous expression of HA-tagged BRCA1 (Supplementary Fig. 4c) substantially restored HR, thus showing that the loss of BRCA1 is a major driver of the HR defect caused by ZC3H18 depletion.

In further experiments, we confirmed that ZC3H18 plays a role in HR by showing that two independent siRNAs reduced ZC3H18 protein, disrupted DR-GFP recombination (Fig. 1a), and blocked the formation of RAD51 foci (Fig. 1b), a key event in HR repair, without disrupting the cell cycle (Supplementary Fig. 1c). Conversely, expression of an siRNA-resistant ZC3H18 rescued the HR defect in ZC3H18-depleted cells (Fig. 1c), indicating that the siRNA effect is due to ZC3H18 depletion. We also demonstrated that ZC3H18-depleted ovarian cancer cell lines (Supplementary Fig. 2a) were sensitive to the DNA crosslinkers cisplatin and melphalan as well as the PARP inhibitors olaparib and veliparib in culture (Fig. 1d, e; and Supplementary Fig. 2b). Consistent with the cell culture results, shRNA-mediated ZC3H18 depletion (Supplementary Fig. 2c) also sensitized xenografted OVCAR-8 cells to olaparib in mice treated with this PARPi (Fig. 1f). Collectively, these results demonstrate that ZC3H18, a gene located in a chromosomal region frequently deleted in HGSOC, is essential for HR and that ZC3H18 depletion sensitizes ovarian cancer cells to platinum agents and PARP inhibitors.

ZC3H18 depletion reduces BRCA1, which drives the HR defect. Because ZC3H18 was previously shown to regulate gene expression through its effects on RNA metabolism, we asked whether ZC3H18 depletion affected expression of genes associated with HR by RNA-seq (Supplementary Data 1). A KEGG pathway analysis showed that multiple HR-associated genes were downregulated (Supplementary Fig. 3), with BRCA1 among the most highly reduced by ZC3H18 depletion (Supplementary Data 1). Because defects in BRCA1 are the most frequent cause of HR deficiency and BRCA1 is a key regulator of HR, we focused the present studies on BRCA1. Consistent with the RNA-seq analysis, ZC3H18 depletion profoundly decreased BRCA1 mRNA (Fig. 2b; Supplementary Fig. 4a) and protein levels (Fig. 2a) in multiple ovarian cancer cell lines and in xenografted OVCAR-8 cells (Supplementary Fig. 2c). Moreover, expression of siRNA-resistant ZC3H18 restored BRCA1 mRNA (Fig. 2c) and protein levels (Supplementary Fig. 4b) in ZC3H18 siRNA-transfected cells confirming that ZC3H18 facilitates accumulation of BRCA1 mRNA and protein. Finally, because multiple HR-associated genes were downregulated by ZC3H18 depletion (Supplementary Data 1 and Supplementary Fig. 3), we next asked whether the loss of BRCA1 was a major contributor to the HR defect caused by ZC3H18 depletion. As shown in Fig. 2d, heterologous expression of HA-tagged BRCA1 (Supplementary Fig. 4c) substantially restored HR, thus showing that the loss of BRCA1 is a major driver of the HR defect caused by ZC3H18 depletion.

ZC3H18 depletion causes BRCA1 promoter hypermethylation. Given that ZC3H18 was previously shown to affect RNA splicing and degradation, we next assessed whether ZC3H18 regulates BRCA1 levels by altering these BRCA1 mRNA processing events. In ZC3H18 siRNA-transfected ovarian cancer cells, we found no evidence of alternative BRCA1 mRNA splicing using a PCR-based method (Supplementary Fig. 5a). Additionally, BRCA1 mRNA half-life was not affected by ZC3H18 depletion (Supplementary Fig. 5b). Similarly, depletion of ZCCHC8, which is a component of the ZC3H18 cap-binding complex that mediates RNA degradation, did not alter BRCA1 mRNA levels (Supplementary Fig. 5c). These results suggest that ZC3H18 mediates BRCA1 expression through a mechanism that differs from its previously identified functions.

Because BRCA1 is frequently silenced by promoter hypermethylation, we next examined whether ZC3H18
depletion affected BRCA1 promoter CpG methylation and the recruitment of DNA methyltransferase 1 (DNMT1), the prototypic DNA methyltransferase, to the BRCA1 promoter. As previously reported, bisulfite sequencing revealed that 10–20% of the BRCA1 promoter CpG sites were methylated in control siRNA-transfected OVCAR-8 cells (Fig. 3a). In contrast, ZC3H18 depletion with two independent siRNAs increased BRCA1 promoter methylation of these sites to ∼50% (Fig. 3a). Consistent with these results, chromatin immunoprecipitation (ChIP) assays showed that ZC3H18 depletion increased the accumulation of DNMT1 on the BRCA1 promoter in two separate HGSOC cell lines, OVCAR-8 and PEA1 (Fig. 3b), without altering DNMT1 expression (Supplementary Fig. 5d). Conversely, treatment of the ZC3H18-depleted cells with the DNA methylation inhibitor 5-aza-2′-deoxycytidine (5-aza-dC) restored BRCA1 mRNA levels without affecting ZC3H18 mRNA levels (Fig. 3c, d), demonstrating that BRCA1 promoter methylation induced by ZC3H18 depletion reduces BRCA1 expression.

ZC3H18 depletion promotes E2F1-mediated repression of BRCA1. To determine how ZC3H18 deficiency induces BRCA1 promoter methylation, we first asked if ZC3H18 associates with the BRCA1 promoter. ChIP of endogenous or overexpressed ZC3H18 demonstrated that ZC3H18 associates with the BRCA1 promoter in multiple ovarian cancer cell lines (Fig. 4a, b), raising the question of whether ZC3H18 depletion promotes E2F1-mediated repression of BRCA1. To address this question, we first asked if ZC3H18 associates with the BRCA1 promoter. ChIP of endogenous or overexpressed ZC3H18 demonstrated that ZC3H18 associates with the BRCA1 promoter in multiple ovarian cancer cell lines (Fig. 4a, b), raising the question of whether ZC3H18 depletion promotes E2F1-mediated repression of BRCA1.
the possibility that ZC3H18 might affect the binding of transcription factors that regulate BRCA1 expression.

E2F family members are key transcription factor regulators of the BRCA1 gene that can either activate or repress transcription, depending on the family member that binds the promoter29–31. E2F1 was previously shown to activate BRCA1 transcription in breast cancer and other cell types30,32,33. Accordingly, we asked whether ZC3H18 affects BRCA1 expression by reducing E2F1 binding to the BRCA1 promoter, an event predicted to reduce BRCA1 expression. Surprisingly, however, ZC3H18 depletion greatly increased E2F1 binding to the BRCA1 promoter (Fig. 4c, d), suggesting that E2F1 was not activating BRCA1 transcription in these cells. Consistent with this possibility, E2F1 depletion did not reduce BRCA1 expression in ovarian cancer cell lines (Fig. 4e; Supplementary Fig. 6a) or in freshly isolated HGSOC tumors from two different mouse PDX models (Supplementary Fig. 6b, c) using primers that are specific for human BRCA1 (Supplementary Fig. 6c), thus demonstrating that E2F1 does not play a major role in BRCA1 transcription in HGSOC.

Although E2F1 is generally considered to be a transcriptional activator, it can also repress transcription in some settings by recruiting the DNA methyltransferase DNMT1 to promoters34. Given that ZC3H18 depletion caused BRCA1 promoter methylation and E2F1 recruitment to the BRCA1 promoter, we reasoned that E2F1 might repress BRCA1 expression in ovarian cancer cells by recruiting DNMT1 when ZC3H18 was depleted. Consistent with this possibility, we found that E2F1 was required for DNMT1 recruitment to the BRCA1 promoter (Fig. 4f). In additional experiments, E2F1 depletion restored BRCA1 expression in ZC3H18-depleted cells (Fig. 4g), further demonstrating that E2F1 mediates BRCA1 repression in this setting.

These findings suggested that the role of E2F1 in BRCA1 transcriptional regulation might differ between ovarian cancer cells, where E2F1 represses BRCA1 transcription (Fig. 4f, g), and breast cancer cells, in which E2F1 promotes BRCA1 transcription30,32,33. To further evaluate this possibility, we examined the effect of depleting E2F1 in MDA-MB-231 breast cancer cells. Consistent with previous reports in breast cancer cells, we found that E2F1 depletion reduced BRCA1 expression in MDA-MB-231 cells (Supplementary Fig. 7a). We next assessed the possibility that ZC3H18 might affect E2F1 and DNMT1 differently in ovarian and breast cancer cells. Indeed, ZC3H18 depletion enhanced E2F1 binding to DNMT1 in ovarian cancer but not in breast cancer cells (Supplementary Fig. 7b, c), suggesting that loss of ZC3H18 leads to E2F1-DNMT1 repressor complex formation in ovarian cancer cells. Despite the different roles of E2F1 in ovarian and breast cancer cells, ZC3H18 depletion reduced BRCA1 levels in both cell lines (Supplementary Fig. 7a). Collectively, these results demonstrate that although ZC3H18 depletion reduces BRCA1 expression in both ovarian and breast cancer cells, the mechanisms underlying ZC3H18 regulation of BRCA1 differ in the two cell types, with ZC3H18 regulating the recruitment of E2F1 and DNMT1 to the BRCA1 promoter to repress transcription in ovarian cancer cells.

ZC3H18 facilitates E2F4 binding to the BRCA1 promoter. Because multiple E2F family members have been reported to regulate BRCA1 transcription in a variety of cell line models15,30,32,33, we next explored the roles of the seven other known family members (E2F2–E2F8) in control and ZC3H18-depleted OVCAR-8 cells. These studies showed that (1) E2F2 was the only E2F family member that affected BRCA1 expression and (2) co-depletion of E2F4 and ZC3H18 did not further suppress BRCA1 mRNA levels (Fig. 5a), suggesting that E2F4 and ZC3H18 are in the same pathway. Additional studies showed that E2F4 depletion with two independent siRNAs reduced BRCA1 mRNA and protein levels in OVCAR-8, PEA1, PEO1, and PEO4 cells without affecting ZC3H18 levels (Fig. 5b; Supplementary Fig. 8a, b). Similarly, E2F4 depletion also reduced BRCA1 expression in short-term ex vivo cultures of HGSOC tumors freshly isolated from three different PDX models (Fig. 5c).

Because the results in Fig. 5a and Supplementary Fig. 8a suggested that ZC3H18 and E2F4 regulate BRCA1 through the same pathway, we next asked whether ZC3H18 and E2F4 affected one another’s interaction with the BRCA1 promoter using ChIP. ZC3H18 depletion reduced E2F4 occupancy on the BRCA1 promoter but did not affect E2F4 expression (Fig. 5d; Supplementary Fig. 8c, d). In contrast, E2F4 depletion did not alter ZC3H18 binding to the BRCA1 promoter (Supplementary Fig. 8e), demonstrating that ZC3H18 enhances the recruitment of E2F4 to the BRCA1 promoter but not vice versa. These findings also suggested that E2F4 might contribute to the effects of ZC3H18 on BRCA1 expression. Consistent with this idea, E2F4 siRNAs increased E2F1 (Fig. 5e) and DNMT1 (Fig. 5f) recruitment to the BRCA1 promoter, blocked HR (Fig. 5g), and sensitized cells to the PARP inhibitor olaparib (Fig. 5h) without disrupting the cell cycle (Supplementary Fig. 8f). Together, these results demonstrate that ZC3H18 enhances E2F4 binding to the BRCA1 promoter, which concomitantly reduces binding of E2F1 and DNMT1 to the promoter and promotes BRCA1 transcription.

ZC3H18 and E2F4 bind adjacent E2F sites. Two key E2F binding sites, E2FA and E2FB15,33, have been identified in the
bidirectional, ~250-bp region that drives transcription of BRCA1 and the opposing gene NBR2 (Fig. 6a). Mutation of either site disrupts BRCA1 promoter activity in ovarian cancer cell lines (Supplementary Fig. 9a), demonstrating that both are required for full transcriptional activation of the BRCA1 promoter in these cells. Based on the observation that ZC3H18 alters the binding of E2Fs to the BRCA1 promoter, we next hypothesized that ZC3H18 directly binds to one of these E2F sites. To test this idea, we performed electrophoretic mobility shift assays (EMSAs) using bacterially expressed, purified ZC3H18 (Supplementary Fig. 9b). These studies showed that ZC3H18 directly binds a BRCA1 promoter fragment with wild-type E2FA and E2FB sites (E2FA/BWT) (Fig. 6b). The specificity of the interaction was confirmed by cold probe competition, supershift assay, and random probe competition (Fig. 6b). Analyses using fragments with mutations in the E2FA (E2FΔA), E2FB (E2FΔB), or both (E2FΔA/B) sites (Fig. 6a) showed that ZC3H18 binds the E2FA site but not the E2FB site (Fig. 6b). In contrast, purified E2F4 binds the E2FB site (Supplementary Fig. 9c, d), whereas purified E2F1 binds to both E2FA and E2FB sites (Supplementary Fig. 9e, f). In agreement with these in vitro observations, we found that E2F4 and ZC3H18 simultaneously occupy the BRCA1 promoter in cells (Fig. 6c, d) using ChIP-Rep-ChIP assays, which can detect the binding of two proteins on a single DNA sequence.

We next investigated how ZC3H18 and E2F4 affect binding of E2Fs to the BRCA1 promoter, using EMSAs and qRT-PCR. Forty-eight hours after transfection, the cells were immunoblotted for the indicated antigens and analyzed for GFP by flow cytometry. Representative immunoblots in Fig. a are from three independent experiments. Unprocessed blots are provided in Source data file. Shown are means ± SEM from three independent experiments in b–d. *p < 0.05, **p < 0.01, ***p < 0.001, unpaired Student’s t-test.
binding. Both ZC3H18 and E2F4 abolished E2F1 interaction with both E2F binding sites (Fig. 6e, f). These results suggest that ZC3H18 binding to the E2FA site — and E2F4 binding to the E2FB site — prevents the interaction of E2F1 with the BRCA1 promoter, thereby blocking E2F1-mediated repression of BRCA1.

**ZC3H18 and E2F4 levels correlate with BRCA1 levels in HGSOC.** Our mechanistic studies discovered that ZC3H18 and E2F4 depletion reduces BRCA1 levels in ovarian cancer cell lines and low-passage, short-term ex vivo-cultured HGSOC PDX models freshly isolated from mice (Figs. 2a, b, 5b, c). To further address whether ZC3H18 and E2F4 affect BRCA1 expression in HGSOC, we compared BRCA1 mRNA levels with ZC3H18 and E2F4 mRNA levels in HGSOC tumors from patients and from PDX mouse models. This analysis showed that BRCA1 mRNA levels were positively correlated with both ZC3H18 and E2F4 mRNA levels in patients (ZC3H18: \( r = 0.19, p = 0.057 \); E2F4: \( r = 0.37, p < 0.001 \)) and PDX models (ZC3H18: \( r = 0.33, p < 0.001 \); E2F4: \( r = 0.34, p < 0.001 \)) (Fig. 7a; Supplementary Data 2). Taken together, these findings suggest that ZC3H18 and E2F4 play a role in regulating BRCA1 expression in HGSOCs.

**Discussion**

As summarized in Fig. 7b, we found that ZC3H18 is a DNA binding protein that regulates BRCA1 transcription by directly interacting with the E2FA site in the BRCA1 promoter. ZC3H18 binding to the E2FA site promotes E2F4 interaction with the adjacent E2F2. Co-occupancy of ZC3H18 and E2F4 on adjacent

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**Fig. 3** ZC3H18 depletion causes hypermethylation of the BRCA1 promoter. a Map of the divergent BRCA1/NBR2 promoter with CpG islands indicated by circles below the map. Methylation patterns obtained by bisulfite sequencing of 10 individual clones of PCR products from genomic DNA of control luciferase (Luc) and ZC3H18 siRNA (siZC3)-transfected OVACR-8 cells. Methylated (filled circles) and unmethylated (open circles) CpG positions are shown. b ChIP assays showing increased DNMT1 occupancy on the BRCA1 promoter in ZC3H18-depleted cells. OVACR-8 (top panel) and PEA1 (bottom panel) cells transfected with control luciferase (Luc) or ZC3H18 siRNAs were harvested 48 h after transfection and processed for ChIP to detect DNMT1 on the BRCA1 promoter. c and d OVACR-8 (c) and PEA1 (d) cells were transfected with control (Luc) or ZC3H18 siRNAs and treated with vehicle or 5-aza-2′-deoxycytidine (5 μM) for 3 days. BRCA1 mRNA (top panel) and ZC3H18 mRNA (bottom panel) levels were analyzed by qRT-PCR. The mRNA levels are normalized to GAPDH mRNA levels as the internal control. Data are means ± SEM from three independent experiments in b–d. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \), unpaired Student’s t-test
E2F sites prevents E2F1 binding to either E2F site and promotes BRCA1 transcription. In contrast, when ZC3H18 is depleted, the E2FA and E2FB sites are instead occupied by E2F1, which represses BRCA1 by recruiting DNMT1 and causing CpG hypermethylation at sites previously found to reduce BRCA1 expression in patients6,27,36,37. In agreement with these observations, loss of ZC3H18 reduces BRCA1 levels, disrupts HR, and sensitizes ovarian cancer cells to the DNA crosslinking agents, cisplatin and melphalan, as well as the PARP inhibitors veliparib and olaparib.

The E2F family of transcription factors consists of eight genes (E2F1-8) that encode nine different proteins38. E2F1, E2F2, and E2F3A can activate or repress transcription depending on whether they interact with pocket proteins, such as retinoblastoma (Rb), p107, and p130 that recruit DNMTs and other enzymes that silence target genes32,34. In contrast, E2F3B, E2F4, E2F5, E2F6, E2F7, and E2F8 were originally classified as repressors38; however, additional studies have shown that E2F4 also activates transcription of multiple genes29,39. The roles of E2F family members in BRCA1 regulation have been primarily investigated in breast cancer cell lines15,30,32,33. Consistent with the typically accepted roles of E2F1 and E2F4, these studies have generally concluded that E2F1 activates BRCA1, whereas E2F4 represses BRCA1 transcription. In contrast, our studies in multiple ovarian cancer cell lines found that E2F1 represses BRCA1 expression, and that this repression requires the DNA
methyltransferase DNMT1 and is correlated with CpG hypermethylation of the \textit{BRCA1} promoter.

We also found unexpected results with E2F4. Using an siRNA screen, we found that (1) of all the E2F family members, only E2F4 depletion reduced \textit{BRCA1} expression in OVCAR-8 cells; (2) \textit{BRCA1} expression in multiple ovarian cancer cell lines was reduced by two independent E2F4 siRNAs; (3) E2F4 depletion in short-term ex vivo cultures of freshly isolated HGSOC tumors from mouse PDX models reduced \textit{BRCA1} expression; and (4) E2F4 expression is positively correlated with \textit{BRCA1} expression in primary HGSOCs and ovarian cancer PDXs. Surprisingly, however, E2F4 was shown to repress \textit{BRCA1} expression in breast cancer and other cell lines\textsuperscript{32}. While we do not currently understand the underlying mechanism for this alternative regulation, E2F4 is converted into an activator when Rb family members are lost\textsuperscript{40}. Accordingly, we speculate that differential expression of Rb family members and/or posttranslational modifications of Rb that regulate interactions with E2F family members may contribute to
the disparate regulation of BRCA1 in ovarian versus breast cancer. Taken together these results suggest that E2F4 is an activator of BRCA1 transcription in ovarian cancer cells.

Notably, the present studies also uncovered a mechanism by which ZC3H18 regulates gene expression, namely that ZC3H18 is a DNA binding protein that interacts with a specific site in the BRCA1 promoter. These findings add to the complex array of functions already ascribed to ZC3H18. These include activating the transcription factor NF-kB via an unknown mechanism14, regulating RNA metabolism by participating in mRNA splicing and export from the nucleus15, and targeting RNA for exosome-mediated degradation16. However, because (1) ZC3H18 depletion did not affect BRCA1 mRNA splicing or stability (Supplementary Fig. 5a, b) and (2) ZCCHC8 depletion, which disrupts the CBCN complex, did not affect BRCA1 levels (Supplementary Fig. 5c), it is unlikely that ZC3H18 is regulating BRCA1 expression by altering the metabolism of BRCA1 RNAs. Instead, our results demonstrate that ZC3H18 directly binds DNA to activate BRCA1 transcription. Consistent with our findings, while this manuscript was in preparation, Winczura et al.17 reported that ZC3H18 depletion reduced the transcription of a subset of genes, including BRCA1. Using ChIP assays, they also showed that ZC3H18 associates with the BRCA1 promoter; however, the mechanism by which ZC3H18 increased BRCA1 expression was not identified. Here we have identified a key mechanism by which ZC3H18 regulates the BRCA1 promoter by showing that ZC3H18’s ability to bind directly to the BRCA1 promoter and regulate the association of E2F family members is a major driver of BRCA1 expression in ovarian cancer. However, as shown in Fig. 2a, b, ZC3H18 has a greater effect on BRCA1 protein levels than on its mRNA levels, suggesting that ZC3H18 may also regulate BRCA1 posttranscriptionally.

The findings presented here raise the possibility that ZC3H18 loss contributes to HR defects by reducing BRCA1 expression. Consistent with this possibility, deep ZC3H18 deletions and low ZC3H18 mRNA levels are nearly mutually exclusive with BRCA1 driver mutations and deep deletions in ovarian tumors analyzed by The Cancer Genome Atlas research network (Supplementary Fig. 10). This correlation was also observed in breast cancer (Supplementary Fig. 10), consistent with our observations that ZC3H18 regulates BRCA1 in breast cancer cells. Accordingly, our findings suggest that loss and/or decreased ZC3H18 expression may help identify HGSOCC patients most likely to benefit from PARP inhibitor and platinum-based therapies.

**Methods**

**Cell lines, cell culture, and small molecules.** The OVCA-8 and OVCA-5 cells were kind gifts from D. Scudierio (NCI, National Institutes of Health). OVCA-8-DR-GFP cells transfected with pcJAScel plus indicated siRNAs were analyzed for GFP fluorescence by flow microfluorimetry 48 h after transfections. HR efficiencies were normalized to control (Luc) siRNA-transfected cells. OVCA-8 cells were transfected with control luciferase (Luc), E2F4, or BRCA1 siRNAs were analyzed by ChIP for E2F4 bound to the BRCA1 promoter. OVCAR-8 cells transfected with control luciferase (Luc) or ZC3H18 siRNAs were analyzed by ChIP for E2F4 bound to the BRCA1 promoter. e, f Depletion of E2F4 or ZC3H18 promotes E2F1 and DMT1 occupancy on the BRCA1 promoter. OVCAR-8 cells transfected with Luc, E2F4, and ZC3H18 siRNAs were analyzed by ChIP for E2F1 (e) and DMT1 (f) accumulation on the BRCA1 promoter. g E2F4 depletion disrupts HR. OVCAR-8-DR-GFP cells transfected with pcJAScel plus indicated siRNAs were analyzed for GFP fluorescence by flow microfluorimetry 48 h after transfections. HR efficiencies were normalized to control (Luc) siRNA-transfected cells. h OVCA-8 cells were transfected with control luciferase (Luc), E2F4, or BRCA1 siRNAs. Forty-eight hours later, the cells were trypsinized, re-plated, and allowed to adhere for 24 h. The indicated concentrations of olaparib were then added, and the cells were cultured for 10 days, stained with Coomassie Blue, and colonies were counted manually. Data are means ± SEM of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, unpaired Student’s t-test. Representative immunoblots in b are provided from three independent experiments. Unprocessed blots are provided in Source data file. The graph in h represents one of three independent experiments that gave similar results. Error bars are standard deviation of triplicate wells from a representative experiment.

**Plasmids and transfections.** Human ZC3H18 cDNA (Dharmacon, MHS6278-20753901), E2F4 cDNA (Addgene plasmid #10914), and E2F1 cDNA (Addgene plasmid #24225)44 were subcloned into the pSFB vector that contains in-frame N- and C-terminal S-peptide, FLAG, and streptavidin-binding peptide tags45. Luciferase reporter assays used pBRC-FF, which contains the BRCA1 promoter driving directly luciferase expression (a kind gift from Dr. Peter Glazer, Yale University)15. To introduce E2F4 and/or E2F2 site mutations into pBRC-FF, PCR-based site-directed mutagenesis was performed using the following: for E2F4 site mutation, 5′-CCTGATACCCCTGGCACGTTGCAACGGAAAAG-3′ (sense) and 5′-CTTTCCGTTGCCACGTGTGCCAAGGGGCTACCG-3′ (antisense); for E2FB site mutation, 5′-CAGGTGCCAAGGGGAAACCGGAGCTGTCGCTTACAGATAAATTAA-3′ (sense) and 5′-GAGAAGUCACGCUAUGAGA-3′ (antisense). The OVCAR-8 and OVCAR-5 cells were from Selleck Chemicals. Melphalan, velparib (ABT-888), and olaparib (AZD2281) were kind gifts from D. Scudierio (NCI, National Institutes of Health). OVCAR-8 cells were transfected with control luciferase (Luc), E2F4, or BRCA1 siRNAs. Forty-eight hours later, the cells were trypsinized, re-plated, and allowed to adhere for 24 h. The indicated concentrations of olaparib were then added, and the cells were cultured for 10 days, stained with Coomassie Blue, and colonies were counted manually. Data are means ± SEM of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, unpaired Student’s t-test. Representative immunoblots in b are provided from three independent experiments. Unprocessed blots are provided in Source data file. The graph in h represents one of three independent experiments that gave similar results. Error bars are standard deviation of triplicate wells from a representative experiment.
methosulfate (PMS) colorimetric assay (Promega) according to the supplier’s protocol. For clonogenic assays, 48 h after siRNA transfection, OVCAR-8 cells were seeded in 6-well plates at 300 cells/well (in triplicate per assay point) and allowed to adhere overnight. The cells were then treated with the indicated concentrations of olaparib, and cultured in the presence of the drug for 8–10 days. Colonies were stained with Coomassie Blue, and colonies of >50 cells were counted manually. Inhibition of colony formation was presented as percentage of colonies formed compared to corresponding untreated control.

Cell cycle analysis. Forty-eight hours after siRNA transfection, cells were harvested, fixed with ethanol, and cell cycle analysis was conducted by flow cytometry following staining of the DNA with propidium iodide.

Immunocytochemistry. Forty-eight hours after siRNA transfection, cells were harvested and plated onto 8-well chamber slides (ThermoFisher Scientific), and the cells were allowed to attach for another 24 h. The cells were then irradiated with 2...
Gray of ionizing radiation, incubated at 37 °C for 6 h, fixed with 3% paraformaldehyde in phosphate buffered saline (PBS) for 12 min, and permeabilized in 0.25% Triton X-100 in PBS for 10 min. The slides were blocked with 3% bovine serum albumin in PBS containing 0.25% Triton X-100, incubated at room temperature overnight with rabbit polyclonal primary antibody to RAD51 (1:250, PC-130, Calbiochem) and mouse monoclonal antibody to phospho-histone H2A.X (γ-H2A.X) (1:250, 05-636, Millipore), washed three times with PBS, incubated with anti-rabbit Alexa Fluor® 488 and anti-mouse Alexa Fluor® 594–conjugated secondary antibodies (1:500; ThermoFisher Scientific) for 1 h in the dark, washed once with PBS, incubated with Hoechst-33342 (1:1000, ThermoFisher Scientific) for 2 min to stain the nuclei, mounted in Prolong Gold Antifade (ThermoFisher Scientific), and examined with a confocal laser scanning microscope using a ×40 or ×100 objective.

**Immunoblotting.** Two days after siRNA transfection, cells were harvested and lysed in 50 mM HEPES (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 mM NaF, 30 mM sodium pyrophosphate, 1 mM Na3VO4, 10 mM 2-mercaptoethanol, 10 μg/mL leupeptin, 5 μg/mL aprotinin, 5 μg/mL pepstatin, and 20 mM microcystin-LR. Immunoblotting was done using the following primary antibodies: rabbit polyclonal ZC3H18 (1:1000, A304-682A, Bethyl Laboratories Inc.); mouse monoclonal BRCA1 (1:2000, sc-6954, Santa Cruz Biotechnology); mouse monoclonal E2F1 (1:500, ab4070, Abcam); rabbit polyclonal E2F4 (1:1000, NBP1-21374, Novus Biologicals); mouse monoclonal DNMT1 (1:5000, ab13537, Abcam); mouse monoclonal HSP90 (Toft, Mayo Clinic, H9010), and rabbit monoclonal HA-tag (1:1000, CST-3724S, Cell Signaling Technology). Secondary antibodies used were: horseradish peroxidase-conjugated anti-mouse immunoglobulin G (1:2000 for BRCA1 and 1:10,000 for all other primary antibodies, 7076 S, Cell Signaling Technology) and anti-rabbit immunoglobulin G (1:16,000 for ZC3H18 and 1:10,000 for all other primary antibodies, 7074 S, Cell Signaling Technology).

**HR assays.** HR assays were performed using OVCAR-8-DR-GFP cells. The cells were transfected twice. On day 1, they were transfected with siRNAs only. On day 2, they were transfected with the same siRNAs as on day 1 along with 20 μg pCßASceI plasmid (encoding I-SceI) with empty vector (pcDNA3) or expression vectors encoding E2F4, BRCA1, or ZC3H18. On day 4, the cells were fixed and stained with Hoechst-33342 to visualize the nuclei, and examined with a confocal laser scanning microscope using a ×40 objective.
vectors for SFB-ZCH3H18 or HA-BRCA1. GFP fluorescence was assessed by flow cytometry on day 5.

**RNA extraction, cDNA synthesis, and quantitative real-time PCR (qPCR).** Total RNA was extracted from frozen cells using the miRNeasy mini kit (Qiagen) following the supplier’s instructions. The extracted RNA was converted to cDNA using oligo(dT) primers and SuperScript™ III reverse transcriptase (ThermoFisher Scientific). Quantitative PCR was performed in triplicate for each sample on a CFX96 real-time PCR system (Bio-Rad) using iTaq Universal SYBR Green Supermix (Bio-Rad). Expression was normalized to GAPDH. The qPCR primers used were:

**BRCA1:**
- Forward: 5′-GCCAAGGCAAGATCTAGAGG-3′
- Reverse: 5′-GTTGCCAACAGGAGCTGA-3′
- ZCH1H8:
- Forward: 5′-TCCGGTCTTCATCTACAG-3′
- Reverse: 5′-CCGGCTCTTCATGACTTC-3′
- E2F4:
- Forward: 5′-CATAGGGGGGAGTTGCCTGT-3′
- Reverse: 5′-CTAAGAGGCGCAGAAGTTG-3′
- ZCHC08:
- Forward: 5′-TGTCACACACACACTGTGTA-3′
- GAPDH:
- Forward: 5′-GAATGGGAGTGGCATTGATG-3′
- Reverse: 5′-AATGATGGAAGGTGATTGAG-3′

**Bisulfite sequencing.** Cells were harvested 48 h after siRNA transfection, and genomic DNA was extracted using phenol-chloroform. Genomic DNA (2 µg) was used for bisulfitization using the EpiMark® Bisulfite Conversion Kit (New England Biolabs) according to the supplier’s protocol. Bisulfite-modified DNA (40 ng) was amplified using Taq DNA polymerase and primer pairs (see below) to cover the BRCA1 promoter (GenBank Accession No. U37574). The PCR products were run on a 1% agarose gel, excised, extracted using QIAquick Gel Extraction Kit (Qiagen), and subcloned into pCR® 2.1-TOPO® TA vector using TOPO® TA Cloning® Kit (ThermoFisher Scientific). At least 10 clones of each PCR product were subjected to Sanger sequencing, and PCR methylation was analyzed by Quantification Tool for Methylation Analysis (QUMA)18. PCR primers specific for bisulfite-converted BRCA1 promoter were designed using MethPrimer software19. The positions of the primers are shown in Fig. 3a.

**Promoter region 1422–1967:**
- Forward: 5′-AGATTTGAGGTGTTAATTAGGTATTCT-3′
- Reverse: 5′-ATAATAATCCTCCCTTAAACATATTTG-3′

**Chromatin immunoprecipitation (ChIP) assays.** Cells (1 × 10⁷) in 15-cm dishes were cross-linked with 1% formaldehyde in media for 10 min at room temperature, and the unreacted formaldehyde was quenched by the addition of one-tenth volume of 0.25 M glycine (pH 7.0). The cells were washed with PBS, dislodged by scraping, collected by centrifugation at 800 × g for 5 min at 4 °C, resuspended in 0.8% ice-cold PBS, and washed on ice for 15 min. The pellet (chromatin) was digested with micrococcal nuclease (2.5 units/ml; New England Biolabs) for 15 min at 37 °C and sonicated for 10 s (sonicator probe D300; Sonics and Materials Inc., Danbury, Connecticut, USA) to aRpc H-EpRCE A440 to 3 to 38 min at 4 °C. The culture was transferred to a 16 °C shaking incubator, 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and the culture was incubated overnight. The cells were harvested by centrifugation, resuspended in PB (50 mM sodium phosphate, pH 7.4, 300 mM NaCl) containing 1 mM imidazole and sonic-clasted, and the lysates were cleared by centrifugation at 14,000 × g for 30 min at 4 °C and incubated with Ni-NTA His-Bind Superflow beads (Novagen) for 1 h at 4 °C. The beads were washed 3 times with PB containing 1 mM imidazole and eluted with PB containing 150 mM imidazole (pH 7.4) at 4 °C. The eluate was then exchanged into NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.5% NP-40, and 1 mM EDTA) using an Amicon® Ultra-15 centrifugal filter unit system (Sigma) and incubated with anti-FLAG M2 antibody (F3165, Sigma) and protein G (ThermoFisher Scientific) for 2 h at 4 °C. The beads were washed five times with NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.5% NP-40, and 1 mM EDTA) and eluted with 20 µg 3xFLAG peptide (Sigma) by centrifugation at 2000 × g for 3 min at 4 °C.

To produce E2F4 and E2F1 proteins, pSFB-E2F4 or pSFB-E2F1 plasmids (40 µg/μL) were electroporated into K362 cells. Cells were harvested 24 h after transfection, and lysed with RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0). The clarified lysate was incubated with anti-FLAG M2 antibody and protein G affinity resin (ThermoFisher Scientific) for 4 h, washed 5 times in RIPA buffer, and SFB-tagged proteins were eluted with 20 µg 3xFLAG peptide in elution buffer (50 mM Tris, pH 7.4, 15 mM MgCl₂, 150 mM NaCl). The purity of the eluted proteins was determined by Coomassie Blue (Bio-Rad) or SYPRO® Ruby staining (ThermoFisher Scientific) in accordance with supplier’s instructions.

**Electrophoretic mobility gel-shift assay.** Complementary 50-mer single-stranded oligonucleotides corresponding to the BRCA1 promoter with wild-type E2F2 and E2F2 sites or mutations in E2F2, E2F2, or both E2F and E2F2 sites were synthesized by Integrated DNA Technologies. The complementary oligonucleotides were end-labeled with [γ-32P]ATP using polynucleotide kinase (NEB), and purified using CHROMA Spin Columns (Takara). Equimolar concentrations of each oligonucleotide were diluted into annealing buffer (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM MgCl₂) in a microcentrifuge tube, heated to 100 °C in a water bath, and cooled slowly to 4 °C to anneal the oligonucleotides. EMSA assays were performed by incubating the duplexed oligonucleotides in gel-shift buffer (20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM MgCl₂, 0.5 mM dsDNA, and 1 mM dithiothreitol) and various concentrations of purified ZCH3H18, E2F1, and/or E2F4 proteins for 20 min at room temperature. For cold competition, the binding reaction mixture was pre-incubated with 50-, 200-, or 500-fold excess unlabeled probe for 10 min before adding the labeled probe. For supershift assay, the reaction mixture was pre-incubated with anti-S-tag antibody20,21 which recognizes the N-terminal S-peptide of the SFB-tagged proteins, for 30 min on ice before adding the labeled DNA probe. Samples were run on a 5% native acrylamide gel in 0.25 × TBE (0.0225 M Tris-borate, 0.0005 M EDTA pH 8.0) at 4 °C. The gel was dried, and autoradiography was performed.**
Luciferase reporter assay. OVCAR-8 cells were transfected with BRCA1-promoter firefly luciferase constructs (2 µg/ transfection) and an internal control for transfection efficiency (PRL-SV40 Renilla luciferase reporter construct, Promega, 100 ng/transfection) and plated into 6-well plates. Samples were harvested 24 h after transfection, and luciferase activity was measured using Dual-Glo® luciferase assay system (Promega) following the supplier’s protocol. To control for inter-sample variations in transfection efficiencies, firefly luciferase readouts were normalized to renilla luciferase readouts.

**Ex vivo culture of HGSOC tumor tissues from PDX mouse models.** To obtain short-term, 2D, ex vivo mono-culture layers of tumor cells, HGSOC tissues from PDX mouse models were harvested, minced into 2–4 mm pieces with a sterile scalpel blade, and dissociated using a tumor dissociation kit (Cat. # 130-096-730, Miltenyi Biotec) following the supplier’s protocol. After dissociation, the cells were washed five times with RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin and 100 units/ml streptomycin (Invitrogen), resuspended in RPMI-1640 medium with 10% fetal bovine serum without antibiotics, and electroporated with control luciferase (Luc), E2F1, or E2F4 siRNAs as described. The cells were then plated in 24-well plates in RPMI-1640 supplemented with 10% fetal bovine serum and 100 units/ml penicillin and 100 units/ml streptomycin (Invitrogen). The plates were cultured for 48 h and harvested for RNA extraction and qRT-PCR.

**Analyses of patient and PDX tumors.** Fresh tissues from high-grade serous ovarian, primary peritoneal, or fallopian tube cancers were collected at the time of primary debulking surgery at Mayo Clinic, Rochester from chemotherapy naïve patients who provided written and informed consent. All biospecimens were coded with a patient heterotransplant (PH) number to protect patient identity in accordance with Mayo Clinic Institutional Animal Care and Use Committee. Brieﬂy, the patient was heterotransplanted to female SCID Beige mice (C.B-I.7IcAcI-nu/Ly5c-/-Ly5k-/-, B6.Cg-Faspr<null>/Rag2<null>−/−, male) with 0.1 – 0.3 cc of minced fresh patient tumor tissue mixed 1:1 with McCoy’s media with rituximab23 in a 1-ml syringe and injected intraperitoneally through a 0.15-inch 16-gauge needle. No enzymatic or mechanical tumor dissociation was performed. Mice were monitored by routine palpation for engraftment and when moribund, tumors were snap frozen for subsequent studies. For primary patient samples, surplus tumor tissue in excess of requirements to generate PDXs was also snap frozen for future RNA work.

Total RNA was isolated from tissues collected from 97 patients and 138 non-overlapping PDX from mice using the RNeasy Mini Kit (Qiagen, #74004) according to the manufacturer instructions. Purification of total RNA concentration and purity was determined on a Thermo Scientific NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE). All samples met RNA integrity number and validated Agilent (Agilent Technologies, Santa Clara, CA) criteria.

RNA libraries were prepared according to the manufacturer’s instructions for the TruSeq RNA Sample Prep Kit (Illumina, San Diego, CA, USA). The concentration and size distribution of the libraries were determined on an Agilent Bioanalyzer DNA 1000 chip (Santa Clara, CA, USA). Libraries were loaded onto flow cells at concentrations of 8–10 pM to generate cluster densities of 700,000,000 mm² following Illumina’s standard protocol using the Illumina cBot and cBot Paired End cluster kit version 3. Flow cells were sequenced as 51 × 2 Paired End reads on an Illumina HiSeq 2000 using TruSeq SBS sequencing kit version 3 and SCS version 1.4.8 data collection software. Base calling was performed using Illumina’s RTA version 1.12.2.4. There were ~45 million reads per sample mapped to the human genome, and 21,686 genes were detected. mRNA levels are expressed as RPKM (reads per kilobase per million mapped reads) using the formula: 

\[ \text{count} \times \frac{\text{genomic length}}{\text{transcript length}} \times \frac{1}{0.5} \times \text{Reads} \]

where count is the number of reads mapping to the gene or exon, total reads is the total number of reads mapping to all genes or exons in that sample, and feature length is the length of the gene or exon. Spearman correlation was used to assess correlation between BRCA1 mRNA levels with ZC3H18 and E2F4 mRNA.

**Data availability**

RAN-seq data have been deposited in the GEO accession GSE136533 and also provided as Supplementary Data 1 and 2. The authors declare that all other data supporting the findings of this study are available within the paper and its supplementary information and source data files upon reasonable request.

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Acknowledgements
This work was supported by NIH (R01 CA194498 to L.M.K, R01 CA184502 to S.J.W., and P50 CA136393 to S.H.K), a Foundation for Women’s Cancer Genentech Ovarian Cancer Young Investigator Career Development Award to A.K., the Fred C. and Katherine Andersen Foundation, a Stand Up To Cancer—Ovarian Cancer Research Fund Alliance—National Ovarian Cancer Coalition Dream Team Translational Research Grant (SU2C-AACR-DT16-15) to L.M.K and S.H.K, and a Wallace and Evelyn Simmons Career Development Award for Ovarian Cancer Research to A.K. We thank Dr. Keith Robertson and Joyce Thompson for providing the protocol and guidance for bisulfite sequencing. We thank Drs. Somaira Nowsheen and Min Deng for help with recombinant ZC3H18 protein purification. We thank Dr. Yuichi Machida for allowing us to use his qRT-PCR machine. We also thank Drs. Martin Fernandez-Zapico and Luciana Almada for help with ChIP and luciferase reporter assays. The results shown in Supplementary Fig 10 are in whole based upon data generated by the TCGA Research Network.

Author contributions
A.K., A.L.O., S.J.W., S.H.K and L.M.K designed the studies. A.K., C.J.H., X.H. and M.Z. performed experiments. A.K., E.P.H., D.R.O. and A.L.O. analyzed data. A.K. and L.M.K. wrote the manuscript with input from all the other authors.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41467-019-12610-x.

Correspondence and requests for materials should be addressed to L.M.K.

Peer review information Nature Communications thanks Simon Powell and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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