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Transcriptional regulation of *BRCA1* expression by a metabolic switch

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Abstract

Though the linkages between germline mutations of *BRCA1* and hereditary breast cancer are well known, recent evidence suggests that altered *BRCA1* transcription may also contribute to sporadic forms of breast cancer. Here we show that *BRCA1* expression is controlled by a dynamic equilibrium between transcriptional co-activators and co-repressors that govern histone acetylation and DNA accessibility at the *BRCA1* promoter. Eviction of the transcriptional co-repressor and metabolic sensor, C-terminal-binding protein (CtBP) plays a central role in this regulation. Loss of CtBP from the *BRCA1* promoter through either estrogen induction, RNAi depletion or increased NAD+/NADH ratio results in HDAC1 dismissal, elevated histone acetylation, and increased *BRCA1* transcription. The active control of chromatin marks, DNA accessibility and gene expression at the *BRCA1* promoter by this "metabolic switch" provides an important molecular link between caloric intake and tumor suppressor expression in mammary cells.

Keywords

BRCA1; histone acetylation; epigenetic modifications; tumor suppressor; NADH; CtBP; RB pocket proteins; estrogen

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The worldwide mortality from breast cancer is the second leading cause of death in women and the number one cause of death from cancer in females aged 20–59 1. Individuals harboring germline mutations in the breast cancer susceptibility gene *BRCA1*, carry an 80% lifetime risk of developing breast cancer 2. Though very few cases of non-inherited sporadic forms of breast cancer have been found to be associated with mutation in BRCA1, nearly 40% of these tumors demonstrate a deficiency in *BRCA1* expression 3. Since the majority of

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AUTHOR CONTRIBUTIONS

L-J.D. and A.G.F. performed the experiments. A.D. and D.L.L. helped write the paper and contributed valuable reagents. L-J.D, and K.G. designed experiments and wrote the paper.

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these cases do not show hypermethylation of the *BRCA1* promoter 4, a growing consensus has emerged suggesting that a large percentage of sporadic, non-inherited breast cancers are associated with altered transcriptional regulation of the *BRCA1* gene 3, 5. The human *BRCA1* promoter is bidirectional, controlling divergent transcription of the *BRCA1* and *NBR2* genes 6 and many aspects of its regulation have been extensively studied. In addition to methylation of specific CpG residues and islands within the promoter 7, several groups have demonstrated that the *BRCA1* promoter is regulated by a complex and dynamic array of DNA binding proteins, transcriptional co-activators and transcriptional co-repressors 8–10.

The protein product of the *BRCA1* gene has many important cellular functions including DNA repair, cell cycle regulation, and transcriptional regulation. Accordingly, deficiency in BRCA1 results in accelerated proliferation, aberrant mitosis, increased chromosome instability and tumorigenesis 11, 12. *BRCA1* transcription is regulated by diverse types of environmental stimuli including genotoxic agents, hypoxia, and mitogenic hormone stimulation. The best characterized stimulant of *BRCA1* expression is estrogen, which induces the highest elevations in *BRCA1* mRNA levels, routinely peaking just prior to the onset of DNA synthesis 13, 14. In this way, BRCA1 is thought to provide a feedback control that monitors and restrains the growth and pro-proliferative effects of estrogen in hormone responsive tissues 14–16. Consequently, disruption of this close opposing relationship with estrogen receptor, in combination with decreased genome stability, is believed to account for the remarkably restricted occurrence of inherited *BRCA1*-related malignancies in hormone regulated tissues like breast, ovary and prostate 16.

The transcriptional co-repressor C-terminal binding proteins (CtBP1 and CtBP2) are members of an evolutionally conserved family of proteins that regulates several different cellular functions in vertebrates 17. Over-expression of these proteins has been linked to epithelial-mesenchymal transition in breast cancer, a process whose gene expression profile shares many similarities with the molecular signature of BRCA1-deficient tumors 17–19. CtBP is a homodimer or heterodimer of CtBP1 and CtBP2 that assembles with a diverse array of factors that regulate chromatin structure. These include, the histone deacetylases (HDACs) HDAC1/2, the histone acetyl-transferases p300/CBP, and the histone methyl-transferase G9a 17. Several studies have shown that CtBP can antagonize the expression of multiple tumor suppressors including *CDH1* (E-cadherin), *CDKN2A* (p16) and *PTEN* 17. Most notably, CtBP contains a binding site for NADH that regulates its ability to dimerize, thus establishing CtBP as an important nuclear sensor of cellular metabolic status 20, 21. In this report we demonstrate that CtBP assembles at the *BRCA1* promoter as part of a dynamic multi-component co-repressor complex containing p130, BRCA1 and HDAC1 that represses local histone acetylation at the *BRCA1* promoter and *BRCA1* transcription. Disruption of this complex by estrogen stimulation and/or changes in NAD+/NADH ratio, results in CtBP dismissal, HDAC1 eviction, increased histone acetylation and subsequent increased *BRCA1* transcription from the *BRCA1* promoter. These observations define a direct link between cellular metabolic status and the expression of BRCA1 and suggest that caloric intake may selectively influence the levels of tumor suppressor function in mammary tissues.

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Results

A dynamic co-regulatory complex controls the *BRCA1* promoter

Prior studies have shown that *BRCA1* transcription can be readily induced by exposure to estrogen 13, 14. Treatment of MCF-7 cells with 10 nM estradiol (E2) for 24 h produces a 6–7 fold increase in both mature and unspliced (nascent) *BRCA1* RNA (Fig. 1**a**). By chromatin immunoprecipitation (ChIP) the *BRCA1* promoter shows preloading by a poised RNA polymerase II (Pol II) and p300 histone acetyl-transferase (HAT) complex in the absence of estrogen stimulation, which is consistent with what has been observed for many promoters in recent genome-wide studies 22. Neither p300/Pol II assembly nor activation-associated histone methylation (H3K4Me3) changes significantly from their elevated levels following estrogen stimulation, though binding by the CREB transcription factor increases more notably (Fig. 1**b–d** and Supplemental Fig. S1). Interestingly, in contrast to Pol II, p300, and histone H3K4Me3, there is a significant increase in the levels of both histone H4 and H3 acetylation (Fig. 1**e,f**). These observations suggest that a major regulatory step following estrogen induction at the *BRCA1* promoter involves events linked to increased promoter proximal histone acetylation that occur following the initial recruitment of p300 and the basal transcriptional machinery.

The increase in histone H3 /H4 acetylation at the proximal promoter despite small changes in p300 HAT occupancy suggests that changes in HDAC recruitment may play a role in the estrogen-induced control of *BRCA1* expression. Notably, in addition to their direct interactions with the Rb pocket protein family, HDACs can be recruited in the context of several different types of co-repressor complexes including, Sin3A, NuRD and CtBP 23, 24. Previous studies have shown that the *BRCA1* promoter is negatively regulated by the dynamic assembly of co-repressor complexes containing, E2F-1, E2F-4, Rb and Rb-related pocket proteins (e.g. p130), and BRCA1 9, 10, 25. Each one of these factors, including BRCA1 itself, have the capacity to form complexes with HDACs either directly or through interactions involving the C-terminal binding protein interacting protein (CtIP) 23, 26 17, 27–29. Accordingly, and consistent with increased histone acetylation at the *BRCA1* promoter following estrogen induction, there is a dynamic loss of HDAC1, p130, BRCA1, CtIP, CtBP, E2F-1 and E2F-4 from the *BRCA1* proximal promoter following estrogen treatment (Fig. 2**a–e**). Also, in agreement with the interdependent interactions between these components and *BRCA1* expression, gene depletion of BRCA1 impairs recruitment of CtBP (Supplementary Fig. S2**a**) while gene depletion of E2F-1 impairs both BRCA1 and CtBP recruitment to the *BRCA1* promoter (Supplementary Fig. S2**b,c**). Furthermore, multiple estrogen dependent complexes containing BRCA1, CtBP, E2F-4, p130 and p300 can be detected by co-immunoprecipitation from nuclear extracts derived from E2 treated and untreated MCF-7 cells (Supplementary Fig. S2**d**). Together, these observations indicate that *BRCA1* transcription is regulated by a multi-component co-repressor complex containing CtBP that is linked to HDAC1 through multivalent interactions. Disassembly and dismissal of this complex from the *BRCA1* proximal promoter, following estrogen stimulation, is a major regulatory step that governs *BRCA1* expression.

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Finally, consistent with transcriptional regulation by a post recruitment step, this estrogen induced dismissal of repressive factors is associated with increased assembly of known elongation factors 30 at the *BRCA1* locus including the negative elongation factor (NELF), the eleven nineteen lysine rich leukemia protein (ELL) and the Cdk9 subunit of the positive transcriptional elongation factor b complex (P-TEFb) (Fig. 2**f**). With the exception of NELF, whose assembly at mammalian promoters occurs without traveling with the elongating polymerase 31, 32, both ELL and P-TEFb are recruited to the *BRCA1* locus and show increased distribution into the transcribed region of *BRCA1* in coordination with increased histone lysine 36 trimethylation marks (H3K36Me3) commonly associated with Pol II elongation 30(Fig. 2**f** and Supplementary Fig. S2**e**).

CtBP regulates HDAC1 recruitment and *BRCA1* promoter acetylation

Thus far, the data demonstrate that complexes containing CtBP play a central role in *BRCA1* transcriptional regulation through control of the deposition of chromatin marks at the *BRCA1* promoter in response to environmental stimuli via regulation of HDAC1 recruitment. To test for the role of CtBP in this process, CtBP expression was suppressed by RNAi inhibition (Fig. 3**a**). Silencing of CtBP expression in MCF-7 cells results in a significant increase in both BRCA1 protein and *BRCA1* RNA message (nascent and mature). The increased *BRCA1* transcription following CtBP depletion is associated with loss of HDAC1 from the *BRCA1* promoter and a corresponding increase in histone H3 and H4 acetylation (Fig. 3**b**–3**d**). Notably these changes occur with minimal alteration in either Pol II or E2F-1 occupancy at the *BRCA1* promoter (Fig. 3**e,f**).

CtBP specificity requires chromatin structure at the *BRCA1* promoter

The increased *BRCA1* induction by CtBP depletion is both gene and promoter specific since it has minimal effects on *BLM* expression, inhibits *H2AZ* and *MAD3L* expression, fails to induce the estrogen-responsive *TFF1* (*pS2*) gene (as has been shown previously 33), and has insignificant effect on the divergent transcription of the *NBR2* gene (Fig. 4**a**). CtBP depletion also mimics the functional influences of BRCA1 over-expression 12 by inducing a cell cycle block in G2 phase (Fig. 4**b**). Finally, CtBP depletion also renders the *BRCA1* promoter less responsive to estrogen induction with minimal influence on *TFF1* (Supplementary Fig. S3) and over-expression of CtBP represses *BRCA1* expression without influencing either *TFF1* or divergent *NBR2* transcription (Fig. 4**c**).

As mentioned previously, the *BRCA1* gene is transcribed from a bidirectional promoter 6, 34. Although most bidirectional promoters show highly correlated bidirectional expression 35, the expression at the *BRCA1* promoter is primarily unidirectional in response to CtBP depletion (Fig. 3**a**) and estrogen induction (see below). Therefore, unique aspects of the *BRCA1* promoter sequence and chromatin structure may account for the unidirectionality. These possibilities were tested by transient transfection of a bidirectional *BRCA1* promoter driving firefly luciferase transcription in the direction of the *BRCA1* 1st exon and *Renilla* luciferase transcription in the divergent direction of the *NBR2* 1st exon (Fig. 4**d**). Over-expression of either CtBP or BRCA1 caused bidirectional repression of transcription, suggesting that promoter and direction specific repression of *BRCA1* transcription by CtBP and BRCA1 requires a structural chromatin context at the endogenous *BRCA1* promoter that

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is not recapitulated by transiently transfected DNA constructs (Fig. 4**d**). To test this, cells transiently transfected with the bidirectional reporter were compared to cells in which the reporter was stably integrated (Fig. 4**e**). As shown in Figure 4**e**, return of the *BRCA1* bidirectional promoter to a chromatin context recovers the unidirectional transcriptional response to estrogen induction, thus highlighting the role of chromatin structure in maintaining the fidelity of *BRCA1* transcriptional regulation.

HDAC inhibition mimics *BRCA1* induction by estrogen or CtBP depletion

The mechanism through which CtBP is able to control *BRCA1* transcription involves direct regulation of the local chromatin marks within the *BRCA1* bidirectional promoter by preventing histone H3 and H4 acetylation (Figs. 1**e**,**f**; and Fig. 3**c**, 3**d**) through HDAC1 recruitment (Fig. 2**a**; and Fig. 3**b**). If the key regulatory step in this process is histone acetylation, then a reasonable prediction would be that HDAC inhibition would lead to *BRCA1* transcriptional induction. As shown in Fig. 5**a** this is indeed the case since incubation of MCF-7 with TSA produces a rapid induction of *BRCA1* transcription that occurs much earlier than estrogen stimulation for both mature and nascent RNA transcripts (Supplementary Fig. S4). Also, like estrogen, TSA induction is inhibited by treatment with protein synthesis inhibitors. Notably, the influence of HDAC inhibition is both promoter-specific and directional since neither *TFF1* nor *NBR2* are significantly induced by HDAC inhibition (Fig. 5**a**). Moreover, both estrogen treatment and HDAC inhibition produce nearly identical increases in chromatin accessibility at the *BRCA1* promoter compared to the untreated control (Fig. 5**b**). As expected HDAC inhibition with TSA results in significantly increased histone H3 and H4 acetylation at the *BRCA1* promoter in addition to some alteration in the assembly of HDAC1, BRCA1 and p130 while having insignificant influence on the assembly of CtBP, E2F1 and p300 (Fig. 5**c**). These differences suggest that, although histone acetylation is a major target, additional protein or factor acetylation may also play a role in the stability of the co-repressor complexes assembled at the *BRCA1* promoter (Fig. 5**c** and Supplementary Fig. S5**a,b**). Finally, loss of p300 by RNAi depletion renders the *BRCA1* promoter unresponsive to TSA treatment and blocks the increase in promoter proximal histone acetylation, thus demonstrating that p300 is primarily responsible for the positive influence of HDAC inhibition on histone acetylation and transcription at the *BRCA1* promoter (Fig 5**d** and Supplementary Fig. S5**c**).

CtBP acts as a "metabolic switch" to control *BRCA1* transcription

A very unique and biologically important aspect of CtBP is that it is most active as a dimer and its dimerization is promoted by binding to NAD+ and NADH 17. CtBP has a much higher affinity (>100 fold) for NADH compared to NAD+, and the free cellular concentrations of both NAD species approach their CtBP binding affinities. Because of this, CtBP is thought to act as both a sensor and effector of cellular metabolic status 20. Estrogen treatment is a major form of mitogenic stimulation that increases cellular proliferation in normal mammary tissues and enhances cell cycle entry in breast-derived cell lines like MCF-7 (Supplementary Fig. S6). The elevated respiration associated with increased proliferation causes the NAD+/NADH ratio to rise to meet the increased energy demand due to surges in protein synthesis and DNA replication 36, 37. This is clearly demonstrated by

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comparison of MCF-7 cells stimulated in the presence of estrogen versus TSA, which shows that estrogen stimulation significantly increases the NAD+/NADH ratio (Fig. 6**a**, left). The NAD+/NADH ratio can also be increased by treatment with the glycolysis inhibitor 2-deoxyglucose (2-DG) (Fig. 6**a**, right). Most important is the observation that increases in the NAD+/NADH levels by 2-DG treatment selectively results in increased expression of *BRCA1* mature and nascent RNA while having no effect on *TFF1* expression or divergent transcription from the *NBR2* gene (Fig. 6**b**). Furthermore the 2-DG induction of *BRCA1* transcription is associated with loss of CtBP from the *BRCA1* promoter and a concomitant increase in histone H3 and H4 acetylation (Fig. 6**c–e**) without significant influence on CtBP expression or E2F recruitment (Supplementary Fig. S7). Finally, though hypoxia has been well established to block *BRCA1* transcription in addition to other factors important in the response to DNA damage 9, 38, its influence on estrogen regulated induction of BRCA1 has not been explored. Since, in contrast to acute 2-DG treatment, hypoxia causes a decrease in the NAD+/NADH ratio, it should block estrogen induction of *BRCA1* transcription. As shown in Figure 7**a**, hypoxia produces a selective block to the estrogen induction of *BRCA1* transcription while influencing neither *TFF1* induction nor divergent transcription from the *NBR2* promoter. These compelling findings demonstrate that CtBP functions as a “metabolic switch” at the *BRCA1* promoter that selectively controls the levels of histone acetylation, chromatin structure, and transcription at the *BRCA1* promoter in response to the cell’s metabolic status.

Prior studies that have mapped nucleosome positioning at the *BRCA1* promoter in quiescent and proliferating cells demonstrate that there is a pronounced shift in locally distributed nucleosomes that results in dramatic increases in chromatin accessibility 39. Mapping of the position of the 5′ end or TSS of *NBR2* and *BRCA1* based on their refseq annotation indicates that the intergenic distance between the two *NRB2* and *BRCA1* TSSs is approximately 133 bp, which is less than the 147 bp occupied by a single nucleosome. These observations indicate that the bidirectional promoter shared by *NRB2* and *BRCA1* is effectively occluded by a single, dynamically regulated nucleosome (Fig. 7**b**). Thus a central regulatory event that controls *BRCA1* expression is an active and persistent competition between DNA bound transcriptional complexes and the centrally occluding nucleosome which undergoes cycles of targeted disruption and stabilization by the competing activities of co-activators and co-repressors assembled at the *BRCA1* promoter. This balance ultimately influences the accessibility of the promoter to additional positive regulators of the transcription cycle that drive *BRCA1* expression.

DISCUSSION

The *BRCA1* promoter is known to be regulated by a variety of different stimuli including estrogen stimulation, DNA damage, and hypoxia 9, 10, 40. Each of these processes influence the NAD+/NADH ratio. Estrogen increases the NAD+/NADH ratio secondary to increased respiration due to the proliferative response 37, 41. Conversely hypoxia increases NADH levels. DNA damage consumes NAD+ through PARP-1 and some forms of DNA damage activate the HIPK2 kinase which phosphorylates CtBP resulting in its elimination via the proteasome pathway 42, 43. All of these pathways contribute to upregulation of

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BRCA1 and, consistent with the proposed role of hypoxia and anaerobic glycolysis in promoting tumor formation, suggest a contribution from the downregulation of tumor suppressors in this process 44. The selective inhibition of estrogen induced expression of *BRCA1* by hypoxia suggests a direct role for this form of regulation during tumor progression in patients with estrogen receptor positive metastatic breast cancer. Recent reports that PARP-1 assembles at the *BRCA1* promoter and that its inhibition represses *BRCA1* transcription indicates a potential role for PARP-1 in *BRCA1* regulation. Although we detect occupancy of PARP-1 at the *BRCA1* promoter in MCF-7 cells this assembly is constitutive and does not appear to be influenced by estrogen or HDAC inhibition (Supplementary Fig. S8).

Post-recruitment regulation of *BRCA1* transcription

The *BRCA1* promoter is a member of a unique class of bidirectional promoters. As mentioned previously, nearly all genes in this class contain CpG islands, exclude TATA boxes and are enriched for binding site for Myc, GABPA, E2F-1, E2F-4, and the CCAAT box 35, 45, all of which have been characterized and studied in the bidirectional *BRCA1* promoter 6, 8–10, 25, 34. A second very common feature of bidirectional promoters is their high enrichment in activating histone marks and poised RNA polymerases suggesting that their chromatin structure is generally more open than other gene classes35. This is certainly consistent with the findings in this current study demonstrating that the resting *BRCA1* promoter is already occupied by a poised RNA polymerase II and p300 complex, maintains constitutive histone marks associated with transcriptional activation, and is highly accessible to nuclease digestion in comparison to β-globin in MCF-7 cells. This is also consistent with genome-wide studies that indicate that certain classes of genes containing CpG islands already have destabilized nucleosomes in their proximal promoter and therefore have reduced requirements for chromatin remodeling factors during activation 46. These properties are highly consistent with what we have observed at the *BRCA1* promoter, where a central destabilized nucleosome provides a major means of control of *BRCA1* transcription through regulation of chromatin marks via histone acetylation. The preloading of Pol II and p300 at the *BRCA1* promoter indicates that subsequent post-recruitment steps play an important role in *BRCA1* transcriptional regulation. One such step in the transcription cycle is elongation. Recent studies are beginning to link histone acetylation and the recruitment of HAT activity to transcriptional elongation possibly through recruitment of P-TEFb through factors like bromodomain protein 4 (Brd4) 47, 48, or the 14-3-3 adapter proteins that bind to phospho-acetylated histone tails to enhance recruitment of other HAT activity to targeted promoters 49. The fact that we observe recruitment of both P-TEFb and ELL to the *BRCA1* promoter and transcribed regions following estrogen induction suggests an intimate association between these factors and chromatin modification during estrogen induction. How estrogen induced chromatin modification facilitates elongation events will be an important area to explore in future studies. Another important area in post-recruitment regulation of *BRCA1* will be the role of CtBP in long range changes in chromatin structure at the *BRCA1* promoter. Prior studies indicate that lost spatial interactions between the promoter and terminator region of *BRCA1* following estrogen stimulation may induce *BRCA1* expression 50. Though we do not detect any interaction between CtBP and the

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terminator region of the *BRCA1* locus, a possible role of the CtBP repressor complex in chromatin looping will be an important area for future investigation.

Multiple modes of estrogen stimulation of the *BRCA1* promoter

The precise manner in which estrogen stimulates the *BRCA1* promoter remains a matter of debate. A general consensus is that estrogen stimulates *BRCA1* through an indirect response based on S-phase entry secondary to mitogenic genomic and non-genomic responses caused by estrogen stimulation (e.g. RAS/MAP kinase signaling) 13, 14, 51. Moreover, multiple genome-wide studies of estrogen receptor binding sites by chromatin immunoprecipitation have failed to detect direct binding of ER to the *BRCA1* promoter 52. However several groups have proposed that regulation could involve direct association of estrogen receptor via a binding site with weak homology to an estrogen response element (ERE) in the downstream alternate *BRCA1* promoter (1b) or through tethering to AP1 or aromatic hydrocarbon receptor binding sites 6, 34, 53, 54. Regardless of these disputed points, none of the mechanisms described above for estrogen stimulation are mutually exclusive and all would be subject to titrated regulation by the assembly and release of the co-activator and co-repressor complexes described in this work.

Does CtBP participate in feedback control of estrogen stimulation?

The estrogen receptor and BRCA1 have a very complex relationship in estrogen responsive tissues 16. Estrogen induces proliferation and the activation of BRCA1 functions in a negative feedback loop to control or restrain the effects of estrogen through targeting estrogen controlled genes, many of which are also controlled by p300 coactivation 55. It is therefore reasonable to imagine that NADH consumption, secondary to estrogen induced proliferation, would serve to activate *BRCA1* expression through dismissal of CtBP/HDAC1 complexes from the *BRCA1* promoter. This is consistent with the observation that *BRCA1* expression is highest in proliferating tissues 56. Interestingly, a high percentage of sporadic breast cancers that show decreased levels of *BRCA1* expression also share gene expression profiles that are very similar to those displayed by basal-like subtypes of breast cancer, which express markers normally associated with myoepithelial cells and is the tumor phenotype that most frequently arises in patients with germline mutations of *BRCA1* 3. A feature that is common to the basal-like phenotype is the loss of markers associated with epithelial differentiation and the acquisition of features that promote motility and invasiveness. Notably this BRCA-like phenotype is very similar to that seen in breast-derived epithelia cells undergoing epithelial-to-mesenchymal transition (EMT) 57, a process that is frequently associated with overexpression of CtBP 17. It is therefore likely that CtBP overexpression may play a role in a variety of malignancies by antagonizing the expression of *BRCA1* and other tumor suppressor genes during tumor progression 17.

High caloric intake, estrogen, *CtBP* and *BRCA1*: a perfect storm?

There is a strong correlation between pre and post-menopausal high caloric intake, weight gain and obesity and increased risk for breast cancer 58. The physiological factors associated with increased risk involve elevated levels of extra-gonadal production of circulating and mammary estrogen due to aromatase present in fatty tissues of the breast and

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throughout the body. The elevated expression of estrogen in the context of higher levels of NADH or lower NAD+/NADH levels due to high caloric intake and/or obesity could establish a state where the pro-proliferative effects of estrogen are not completely balanced by the protective functions of BRCA1 that would normally restrain estrogen induced proliferation and heighten genome surveillance. It is reasonable to speculate that the enhanced CtBP activity in mammary tissues with lower NAD+/NADH ratios, secondary to high caloric diet or obesity, may contribute to the increased risk for malignancies of the breast. In this regard, it would also be of interest to ascertain what percentage of postmenopausal breast cancer cases, associated with pre or post-menopausal weight gain or obesity, display the basal-like phenotype associated with *BRCA1* deficiency and/or germline mutation.

MATERIALS AND METHODS

Chemicals and reagents

E2 (estradiol), TSA (trichostatin A), CHX (cycloheximide) were from Sigma Aldrich. 2-DG (2-Deoxy-D-glucose), anti-E2F1, anti-E2F4, anti-p107, anti-p130, anti-CtBP, and anti-CtIP antibodies were from Santa Cruz Biotechnology. Anti-CtBP antibodies are cross-reactive with both CtBP1 and CtBP2. Anti-acetylated histone H3 and anti-acetylated histone H4 antibodies were from Millipore. Anti-HDAC1 antibody was from ABR (Affinity BioReagents). DNase I was from Roche.

Cell culture

MCF-7 cells were maintained in regular DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% (v/v) FBS (fetal bovine serum), penicillin/streptomycin (Invitrogen) and insulin. Prior to treatment, MCF-7 cells were grown in phenol-red free DMEM medium supplemented with 5% (v/v) charcoal filtered FBS, penicillin/streptomycin, 1 mM pyruvate and insulin for at least 3 days. Generally, 10 nM E2, 500 ng ml^{-1} TSA and 10 μg ml^{-1} CHX, were used to treat the cells and 95% (v/v) ethanol was used as a vehicle control. 2-DG was dissolved in ddH_2O and used at 10 mM final concentration.

Chromatin immunoprecipitation

All ChIP experiments were performed as previously described 47, with minor revisions. In brief, cells were crosslinked with 1% formaldehyde (w/v) for 5 min at room temperature. The cross-linking was quenched by 0.125 M glycine for 15 min. Then the cells were washed twice with PBS and collected. Approximately 1×10^7 crosslinked cells, resuspended in 1ml immunoprecipitation (IP) buffer (150 mM NaCl, 50 mM Tris-HCl pH7.5, 5 mM EDTA, 0.5% (v/v) NP-40, 1.0% (v/v) Triton X-100, and freshly added proteinase inhibitor cocktail), were sonicated for 13×20 s with 30 s break. Then the sonicated cells were centrifuged and the supernatant was used for IP. In most cases, the lysate from at least 2 million cells (up to 10 million) was incubated with each antibody overnight with rotation at 4 °C. The pre-blocked protein G beads were added to the lysate with rotation for 2 h at 4 °C. The beads were washed with IP buffer supplemented with 500 mM NaCl, IP buffer and then TE pH8.0 buffer. Finally, the precipitated DNA-protein complex was eluted 10 min at 100 °C with chelex-100 or overnight incubation with SDS and proteinase K at 65 °C and used directly

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for qPCR. Alternatively protein and SDS was removed through standard phenol-chloroform extractions and ethanol precipitation. The qPCR was performed using the SybrGreen® qPCR kit by Invitrogen. The sequences of all primers are provided in the supplemental materials and methods.

Luciferase reporter assays

After 3 days growth in phenol-red free DMEM medium MCF-7 cells were trypsinized, washed twice with PBS and resuspended in DMEM medium with 2.5% FBS (v/v). The plasmid harboring the bidirectional promoter of the *BRCA1* locus driving *Renilla* luciferase transcription from the *NBR2* TSS and firefly luciferase transcription from the *BRCA1* TSS 9 was kindly provided by P.M. Glazer. The CtBP expression vector was purchased from Origene. The BRCA1 and p300 expression vectors were previously described 10. pcDNA 3.1 is from Invitrogen. In brief, approximately 5×10^6 MCF-7 cells were transfected with 6 μg reporter plasmid and 10 μg expression vector or control pcDNA 3.1 empty vector. Electroporation was performed using the ElectrosquarePorator ECM T820 according to the manufacturer's instructions. After electroporation, the cells were again seeded to plates with the regular phenol red-free DMEM medium. By 48 h, the cells were collected for luciferase assay by using a Dual Luciferase Reporter assay system kit (Promega) according to the manufacturer's instructions. Both firefly and *Renilla* luciferase activity were normalized to total protein levels.

Transfection, qRT-PCR and western blotting

MCF-7 cells were grown in phenol-red free DMEM medium for 3 days. The cells were split and seeded to 80% confluency. The transient transfection of CtBP to MCF-7 cells was performed on the next day using Lipofectamine LTX Reagent (Invitrogen). After 48 h, the cells were collected for further assays. The total RNA was prepared using the RNAeasy kit (Qiagen) following the manufacturer's protocol. Reverse transcription of 1 μg RNA was carried out by following the QuantiTect® Reverse Transcription procedure (Qiagen). For western blotting, the cells were resuspended in the lysis buffer (50 mM Tris pH7.5, 1 mM EDTA, 150 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) TritonX-100, 1% (w/v) sodium deoxycholate and freshly added proteinase inhibitor cocktail) for 30 min on ice. The lysates were centrifuged for 20 min at 12,000 × *g* at 4 °C and the supernatants were analyzed by western blotting.

CtBP knockdown

siRNA oligonucleotides specific for CtBP 43 were synthesized by Dharmacon Research, Inc. The Scramble II Duplex was used as a negative control. MCF-7 cells were transfected with 100 nM oligonucleotides and, 48 h later, the expression of CtBP was analyzed by either qRT-PCR or western blotting using anti-CtBP antibody.

NAD+/NADH ratio determination

Determination of the NAD+/NADH ratio in cellular lysates was performed using a Biovision NAD+ and NADH quantitation kit according to the manufacturer's specifications.

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Flow cytometry

For determination of DNA content, all floating and attached cells were collected and combined for analysis. The cells were fixed with cold 70% (v/v) ethanol and stored at −20 °C for at least 24 h. The cells were then washed twice with 1X PBS and once with 1X PBS supplemented with 0.1% (v/v) TritonX-100 and resuspended in 50 μg ml^{-1} PI (propidium iodide) staining buffer in the presence of 300 μg ml^{-1} RNase A for 30 min at room temperature. Flow cytometry was performed using FACScalibur (Becton Dickinson) equipped with CellQuest software (Becton Dickinson).

DNase I hypersensitivity assay

MCF-7 cells were collected from plates by trypsinization and washed twice with ice-cold PBS. To isolate nuclei, cells harvested at 250 × *g* for 5 min at 4 °C were resuspended in ice-cold Buffer A (15 mM Tris-HCl (pH8.0), 15 mM NaCl, 60 mM KCl, 1 mM EDTA, 0.5 mM EGTA, 0.5 mM spermidine, and freshly added proteinase inhibitor cocktail) followed by addition of equal volume of Buffer A containing 0.04% (v/v) NP-40. Nuclei were washed three times with ice-cold Buffer A and resuspended again in Buffer A. For each DNase I digestion, approximately 1×10^6 nuclei were harvested and resuspended in 200 ul of pre-warmed (37 ° C) Buffer A, supplemented with 6 mM $CaCl_2$, 75 mM NaCl, and the DNase I (0, 170, 340, and 680 units). Digestions were performed for 6 min at 37 °C, quenched by addition of stop buffer (50 mM Tris-HCl (pH8.0), 100 mM NaCl, 0.1% (w/v) SDS, 100 mM EDTA, and 50 μg ml^{-1} RNase A) and incubated 1.5 h at 55 °C. Samples were deproteinized at 55 °C overnight in the presence of 50 μg ml^{-1} proteinase K prior to qPCR analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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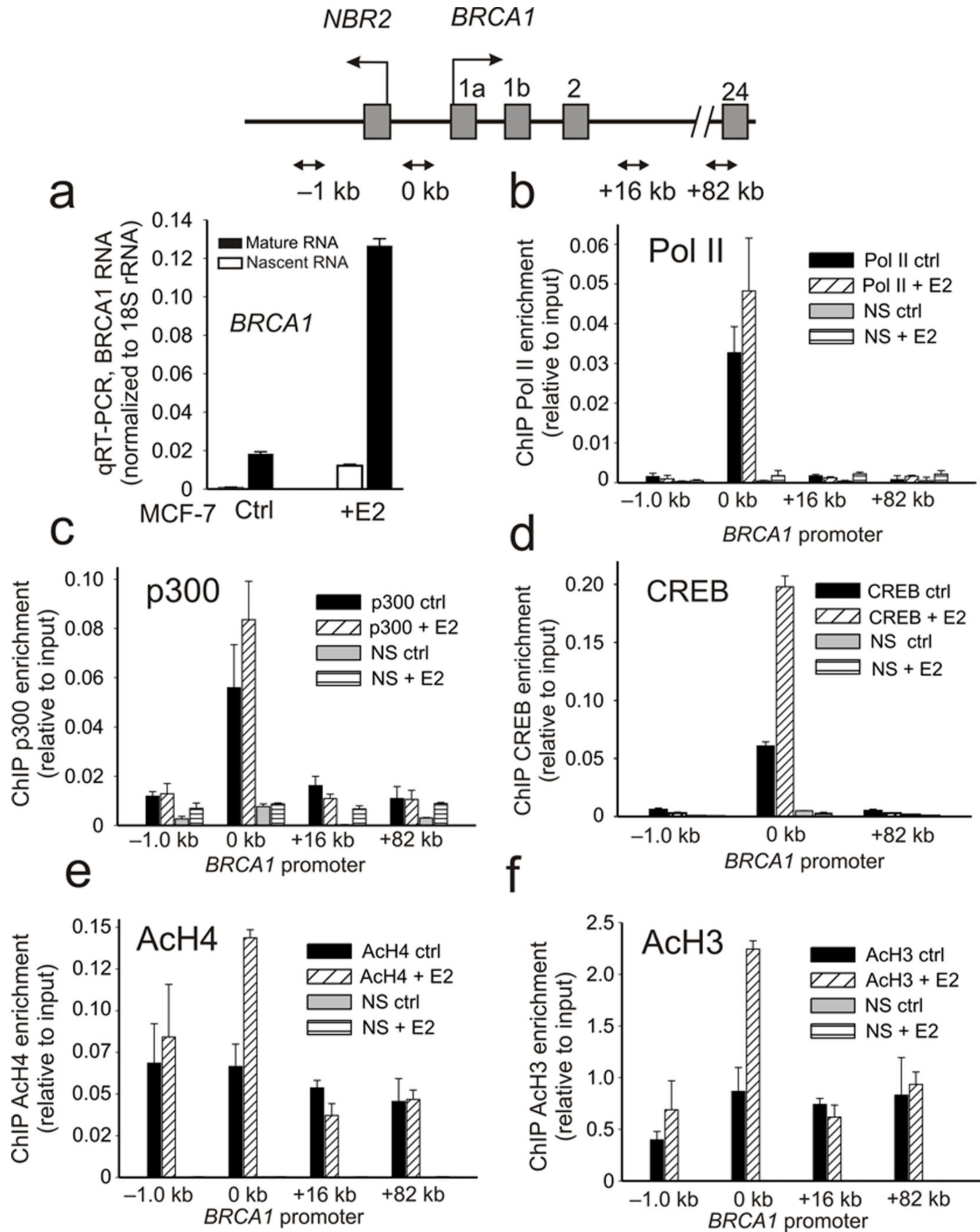

Figure 1.

Estrogen induction increases histone acetylation at the *BRCA1* promoter. Top panel: a schematic illustration of the bidirectional promoter of the *BRCA1*/*NBR2* gene locus showing positions of the ChIP amplicons. (**a**) *BRCA1* nascent and mature RNA expression in control or MCF-7 cell treated 24 h with 10 nM estradiol (E2). Error bars indicate the s.e.m. of N=3 biological replicates. (**b–f**) ChIP profiles of resting and E2 stimulated MCF-7 cells using antibodies against Pol II (**b**), p300 (**c**), CREB (**d**), acetylated histone H4 (**e**), and acetylated

histone H3 (**f**) at the *BRCA1* promoter. Error bars represent the s.e.m. for N=3 (Pol II), N=2 (p300), N=2 (CREB), N=3 (AcH4) and N=3 (AcH3) biological replicates.

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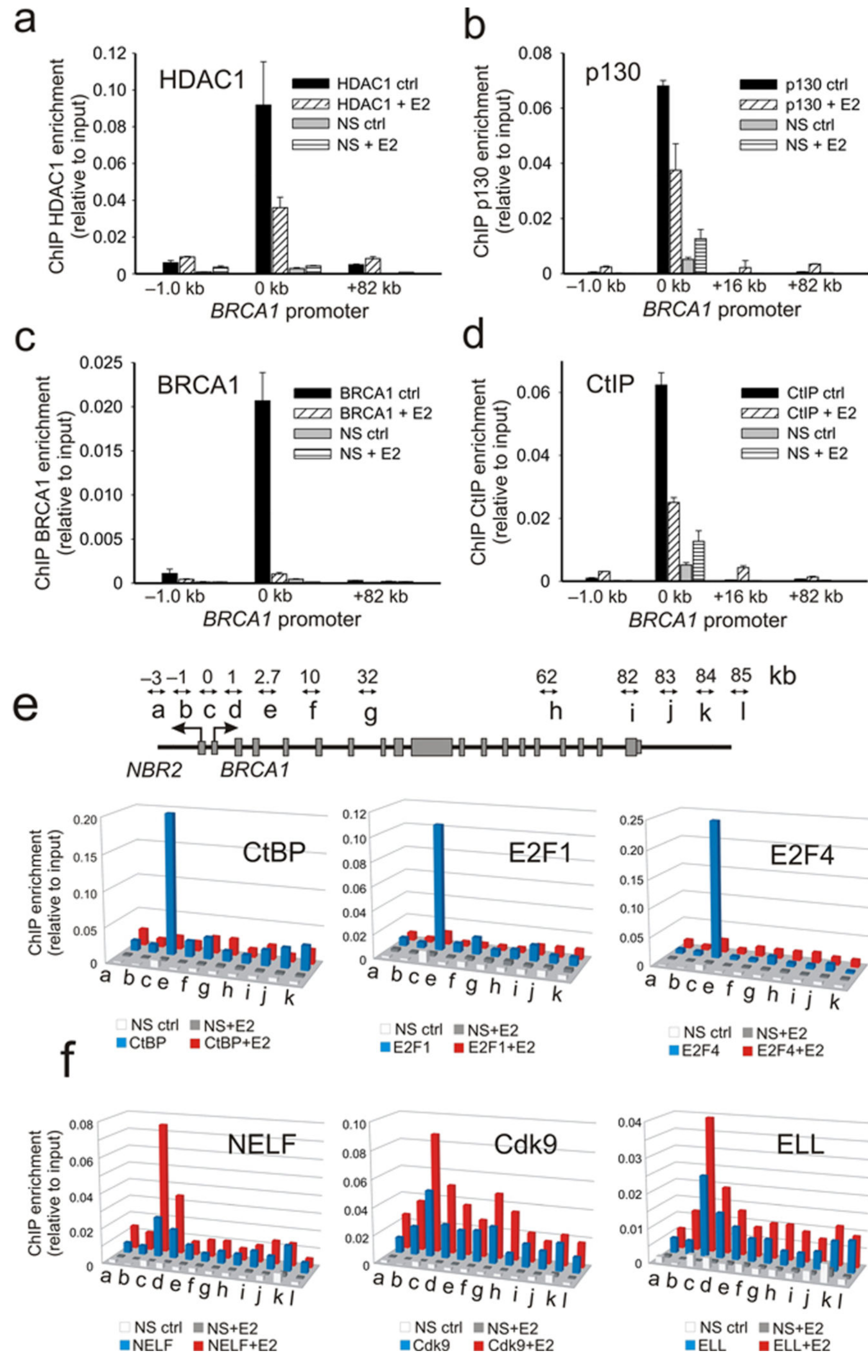

Figure 2.

A multi-component co-repressor complex containing CtBP is dismissed and elongation factors are recruited to the *BRCA1* promoter following estrogen induction. (**a–d**). ChIP profiles of MCF-7 cells stimulated 24 h with E2 using antibodies against HDAC1, p130, BRCA1, and CtIP as indicated. Error bars represent the s.e.m. for N=2 biological replicates. (**e**) Upper panel shows schematic of location of ChIP primer pairs (*a–j*) across the *BRCA1* locus. ChIP profiles of CtBP, E2F1, and E2F4 enrichment across the 85 kb *BRCA1* locus before (blue) and after (red) estrogen induction. The mean of N=2 biological replicates is

represented and is associated with an average s.e.m. that is 24.6% of the mean. (**f**) ChIP profiles of NELF, Cdk9, and ELL enrichment across the BRCA1 locus before and after estrogen induction. The mean of N=2 biological replicates is represented and is associated with an average standard error that is 19.5% of the mean.

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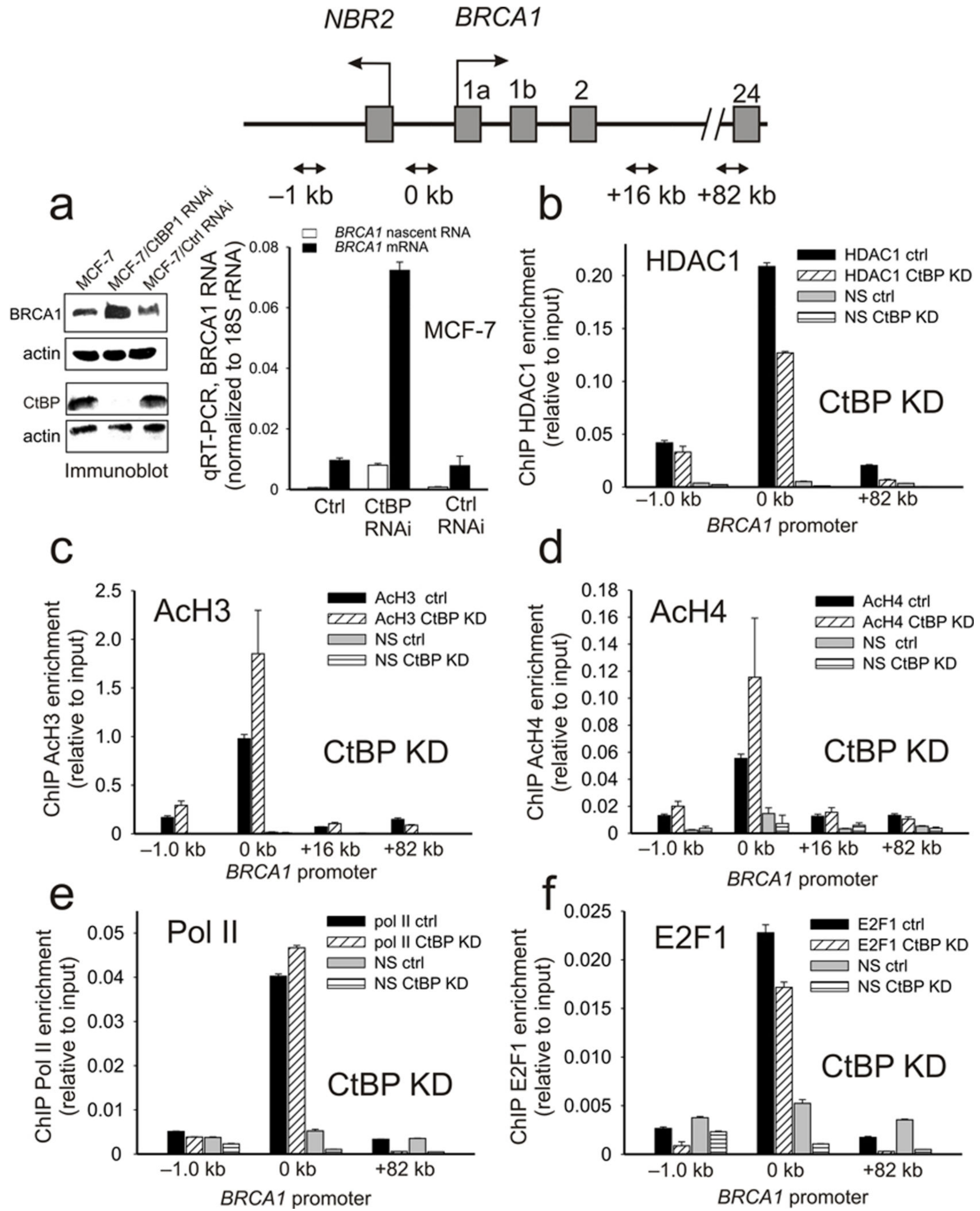

Figure 3.

CtBP regulates *BRCA1* expression by influencing histone acetylation at the *BRCA1* promoter. (**a**) (left panel) Immunoblot of CtBP and BRCA1 expression in control and MCF-7 cells depleted of CtBP by RNAi. Actin is shown as an endogenous control. (right panel) Nascent and mature *BRCA1* RNA levels in control and CtBP depleted MCF-7 cells. Error bars represent the s.e.m. for N=3 biological replicates. (**b–f**) Estrogen stimulated enrichment of HDAC1 (**b**), acetylated histone H3 (**c**), acetylated histone H4 (**d**), Pol II (**e**),

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and E2F1 (**f**) at the *BRCA1* promoter in control and CtBP depleted MCF-7 cells. Error bars represent s.e.m. for N=2 independent biological replicates.

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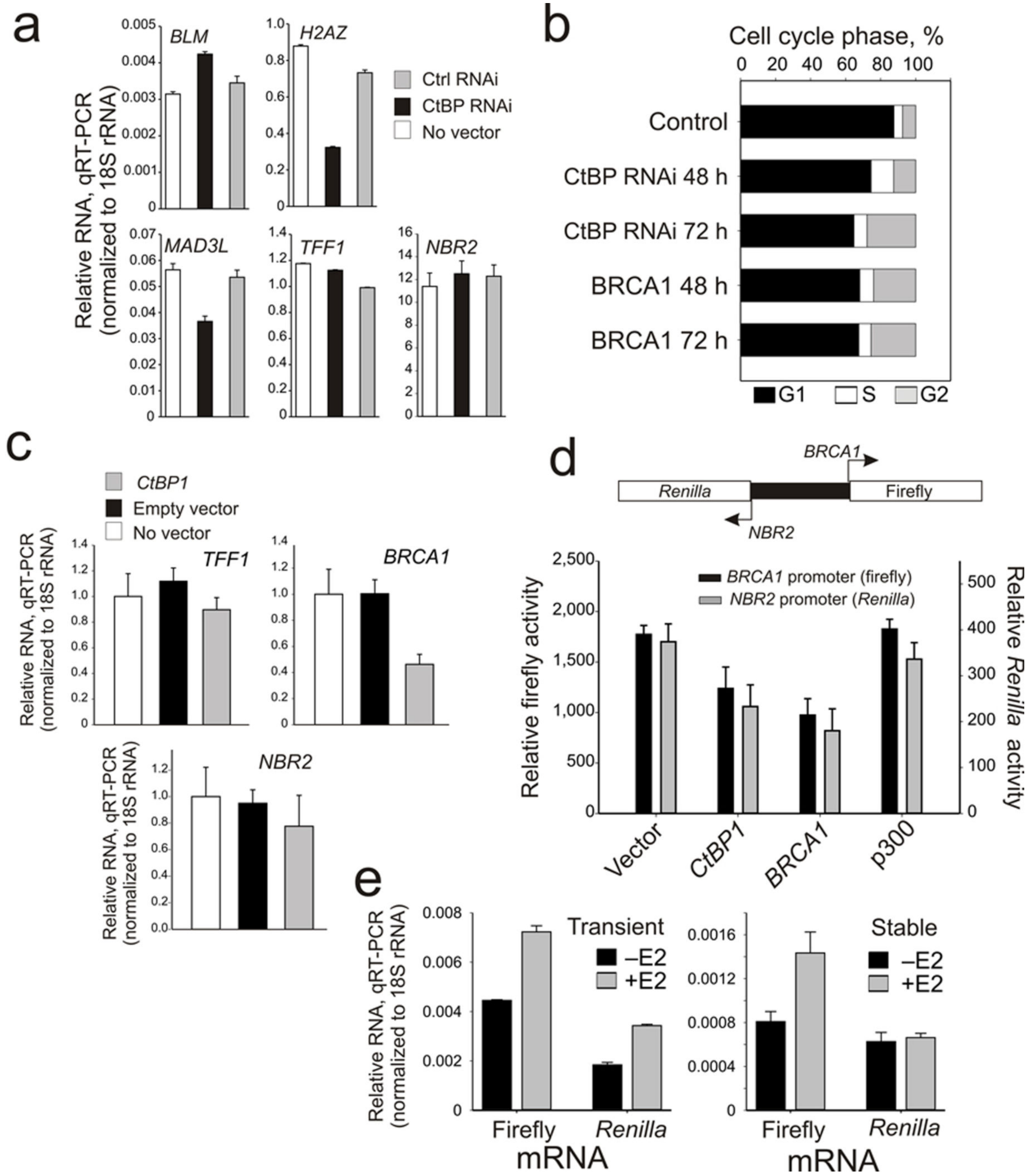

Figure 4.

CtBP control of *BRCA1* is gene specific, functionally influences cell cycle progression and is chromatin dependent. (**a**) mRNA levels of *BLM*, *H2AZ*, *MAD3L*, *TFF1*, and *NBR2* in control and CtBP depleted MCF-7 cells. Error bars represent the s.e.m. for N=3 biological replicates. (**b**) Cell cycle profiles (percent distribution in G1, S, and G2/M phases) of MCF-7 cells depleted of CtBP for 48 h and 72 h, or over-expressing *BRCA1* for 48 h and 72 h. (**c**) mRNA profiles of *TFF1*, *BRCA1* and *NBR2* in control MCF-7 or cells 48 h after transfection with empty vector or CtBP1 expressing plasmids. Error bars represent the s.e.m. for N=3

independent biological replicates. (**d**) Upper panel: schematic diagram of the dual *NBR2*/*BRCA1* promoter reporter. Lower panel: Firefly and *Renilla* luciferase activity profiles of MCF-7 cells co-transfected with the dual *NBR2*/*BRCA1* luciferase reporter and either control or vectors expressing *CtBP1*, *BRCA1* or p300. (**e**) Firefly and *Renilla* luciferase mRNA levels in MCF-7 cells expressing a transiently (left) or stably integrated (right) *BRCA1* bi-directional firefly/*Renilla* luciferase reporter after 24 h stimulation with estrogen. Error bars represent the s.e.m. for N=2 biological replicates.

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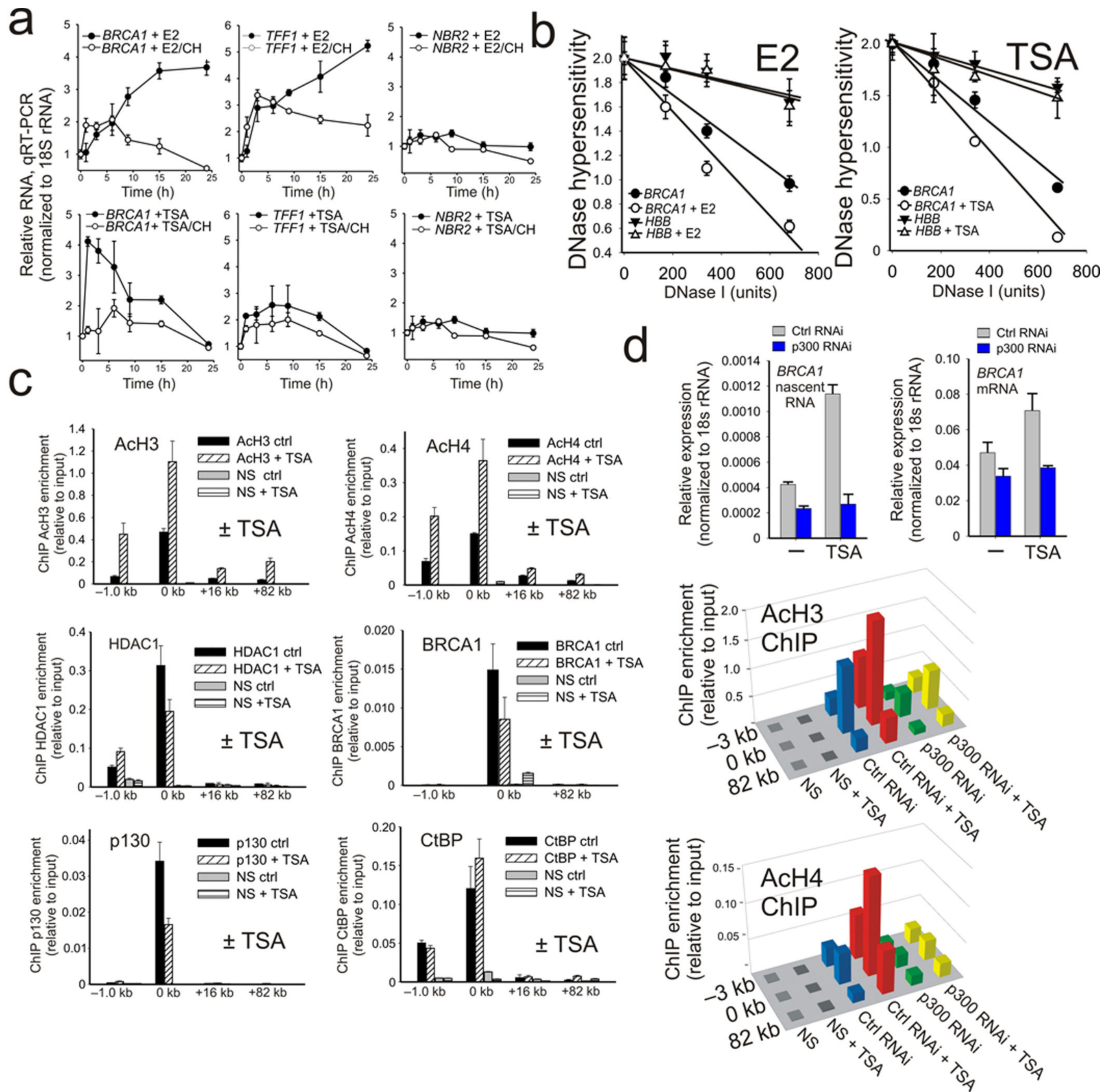

Figure 5.

TSA mimics estrogen induced activation of *BRCA1* by increasing p300 dependent histone acetylation at the *BRCA1* promoter. (**a**) Time course of *TFF1*, *NBR2*, and *BRCA1* expression in MCF-7 cells treated 0–24 h with either E2, E2 + cycloheximide (10 μg ml^{-1}), TSA (500 ng ml^{-1}), or TSA + cycloheximide as indicated. Error bars represent the s.e.m. for N=2 independent biological replicates. (**b**) DNase I hypersensitivity profile of the *BRCA1* promoter and an (*HBB*) locus control from MCF-7 cells treated with either estrogen or TSA. The error bars represent the s.e.m. for N=3 biological replicates. (**c**) Acetylated histone H3,

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acetylated histone H4, HDAC1, BRCA1, p130, and CtBP ChIP profiles at the *BRCA1* promoter in control or MCF-7 cells treated 1 h with 500 ng ml^{-1} TSA. Error bars represent the s.e.m. for N=2 biological replicates. (**d**) Upper panel: TSA stimulated expression of *BRCA1* nascent and mature RNA levels in either control or p300 depleted MCF-7. Error bars represent the s.e.m. for N=2 biological replicates. Lower panel: ChIP enrichment for H3 and H4 histone acetylation at the *BRCA1* locus in control versus p300 depleted MCF-7 cells with or without TSA stimulation. Means from N=2 independent biological replicates are shown.

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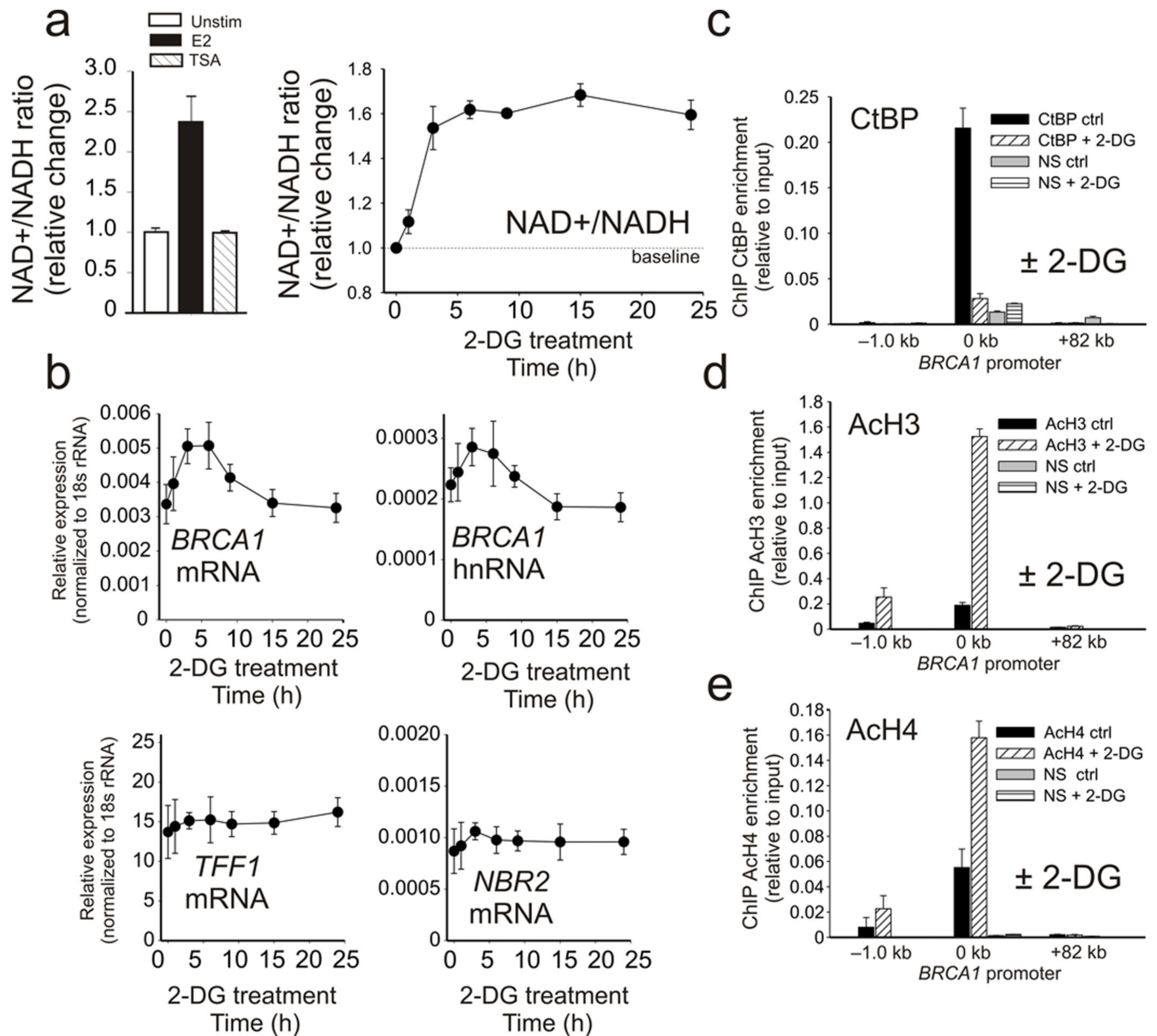

Figure 6.

CtBP functions as a metabolic switch to control *BRCA1* expression. (**a**) (Left) Relative change in the NAD+/NADH ratio in lysates from MCF-7 cells treated with vehicle or E2 for 24 h, or TSA for 1 h as indicated. (Right), Time course of the relative change in the NAD+/NADH ratio in MCF-7 cells treated 0–24 h with 10 mM 2-DG. (**b**) Relative enrichment of *TFF1*, *NBR2*, and nascent and mature *BRCA1* RNA in MCF-7 cells treated 0–24 h with 2-DG. Error bars represent the s.e.m. for N=2 independent biological replicates. (**c–e**). ChIP enrichment for CtBP (**c**), acetylated histone H3 (**d**) and acetylated histone H4 (**e**) at the *BRCA1* promoter in MCF-7 cells treated 3 h with 2-DG. Error bars represent the s.e.m. for N=2 independent biological replicates.

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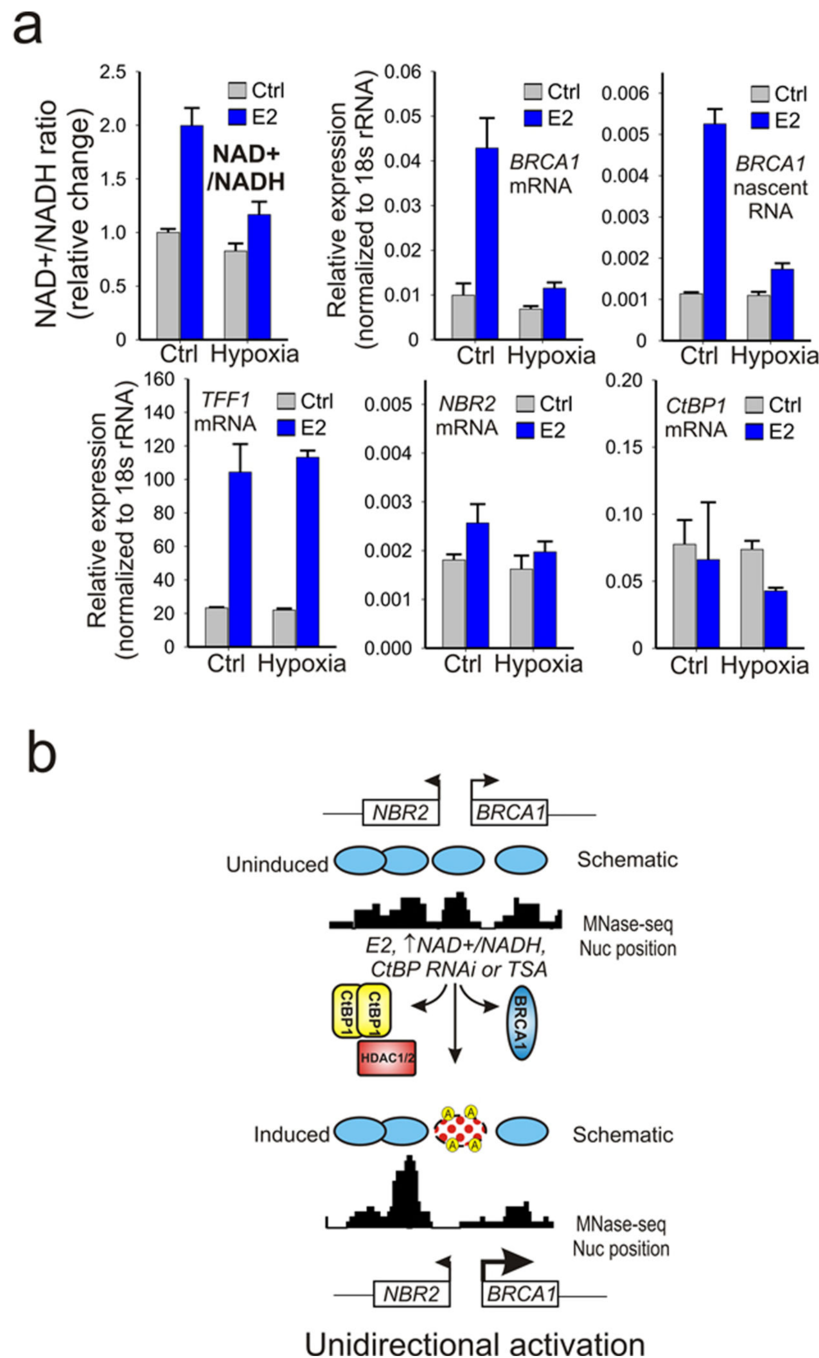

Figure 7.

Hypoxia inhibits estrogen induced changes in the NAD+/NADH ratio and selectively represses estrogen induction of *BRCA1* transcription. (**a**) Assay of relative change in the NAD+/NADH ratio, and *BRCA1*, *TFF1*, *NBR2*, and *CtBP1* expression in control versus hypoxic cells in the presence or absence of estrogen stimulation.(**b**) Schematic hypothetical model for the mechanism of CtBP control of *BRCA1* transcription. The nucleosome positioning is according to Schones et al 2008 39 by the genome-wide sequencing of micrococcal nuclease generated fragments. E2, changing NAD+/NADH ratio, CtBP

knockdown or TSA treatment induce removal or inactivation of a repressive complex composed of CtBP, BRCA1 and HDAC1/2 at the dual *BRCA1* promoter. Acetylation associated destabilization of the centrally positioned nucleosome, in combination with the asymmetric nucleosome distribution at the *BRCA1* locus biases more toward expression of *BRCA1* compared to *NBR2* in response to the activating signals.

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