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## **BRCA1 tumor suppression occurs via heterochromatin mediated silencing**

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### **Abstract**

Mutations in tumor suppressor *BRCA1* lead to breast and/or ovarian cancer. Here we show that loss of *BRCA1* in mice results in transcriptional derepression of the tandemly repeated satellite DNA. *BRCA1* deficiency is accompanied by reduction of condensed DNA regions in the genome and loss of ubiquitylation of histone H2A at satellite repeats. *BRCA1* binds to satellite DNA regions in vivo and ubiquitylates H2A in vitro. Ectopic expression of an H2A fused to ubiquitin reverses the effects of *BRCA1* loss, suggesting that *BRCA1* maintains heterochromatin structure via ubiquitylation of histone H2A. Satellite DNA derepression was also observed mouse and human *BRCA1* deficient breast cancers. Ectopic expression of satellite DNA can phenocopy *BRCA1* loss in centrosome amplification, cell cycle checkpoint defects, DNA damage and genomic instability. We propose that the role of *BRCA1* in maintaining global heterochromatin integrity accounts for many of its tumor suppressor functions.

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Breast cancer susceptibility gene1 (*BRCA1*) was identified as a hereditary cancer susceptibility gene which increased<sup>1</sup> risk for the development of breast and ovarian cancer in *BRCA1* mutation carriers to as high as 95% by age 70<sup>2</sup>. The *BRCA1* protein contains a RING finger domain in the N-terminus with ubiquitin E3 ligase activity and two BRCT repeats in the C-terminus<sup>3</sup>. *BRCA1* is highly expressed in proliferative cells and its loss

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QZ generated and QZ, NT and GMP maintained all the KO animals. GMP and QZ made the initial heterochromatin observation. QZ and AMH performed confocal microscopy experiments. GMP, QZ and NT performed ChIP experiments. RNA isolation and microarray experiments were performed by QZ, GMP and NT. Microdissection of murine brains were performed by GMP and AMH under the guidance of FHG. GMP designed the H2A-ubiquitin fusion experiments that were performed by QZ, GMP and NT. Satellite RNA experiments were designed by GMP and QZ and performed by QZ, GMP and NT. HS established the embryonic neural stem cell isolation and culture. PN obtained, isolated and curated the clinical patient samples, which were analyzed by QZ, GMP and NT. All other experiments were performed by QZ, GMP and NT. All experiments and experimental design was performed under the supervision of IMV. GMP, QZ and IMV wrote the manuscript.

leads most prominently to genetic instability and growth arrest. These observations have implicated *BRCA1* in a multitude of disparate cellular functions, including DNA damage repair, cell cycle checkpoint activation, transcriptional regulation, DNA replication, centrosome function, X inactivation, among others<sup>4</sup>. Presently no unifying mechanistic framework exists to tie the reported biochemical activities of *BRCA1* to its tumor suppressor function<sup>3</sup>.

## Heterochromatic centers in *BRCA1* KO cells

High levels of *BRCA1* expression have been found in the embryonic neuroectoderm<sup>5</sup>, the postnatal cerebellum<sup>6</sup> and the subgranular zone of the dentate gyrus in the adult brain, which are the sites of embryonal and adult neurogenesis, respectively. We initially set out to study the contribution of *BRCA1* to neural stem cell (NSC) proliferation through targeted deletion of *BRCA1* in nestin-expressing NSCs (Supplementary Fig. 1a). As expected, the levels of *BRCA1* protein and RNA are significantly reduced in the brains of *BRCA1* KO mice (Figs. 1a and Supplementary Fig. 1b). To characterize the mutant brains, we performed microarray analyses and found evidence of altered epigenetic regulation in the absence of *BRCA1* as some imprinted genes are deregulated (Supplementary Table 1). Most notably, upregulation of both *Igf2* and *H19* expression has been verified by quantitative RT-PCR experiments using cortical RNA samples (Fig. 2a).

Heterochromatic centers are highly condensed regions of heterochromatin with repetitive tandem DNA, which can be visualized by DAPI staining. In neurons these centers are particularly prominent. We analyzed the nuclei of the mutant brains by DAPI staining and found a marked reduction in the number of heterochromatic foci in the cell, especially in the perinuclear region, when compared to control cortices (4.88 foci/cell to 1.99 foci/cell) (Student's *t*-test,  $p < 0.0005$ ) (Fig. 1b). Moreover, the size of the heterochromatic foci appeared to be larger and diffuse in mutant brains. These observations suggest a defective heterochromatic structure in *BRCA1*-deficient cells. To study the nature of this defect, we stained the nuclei of cortical cells with heterochromatin protein (*HPI*)<sup>7</sup>, a bona fide pericentric heterochromatin associated protein, and found that *HPI* localized to heterochromatic centers. The number of *HPI*-positive foci in mutant cortices was decreased from 2.86 foci/cell to 2.13 foci/cell (Student's *t*-test,  $p < 0.0005$ ) (Fig. 1c, top row and Supplementary Fig. 1c, left panel). Moreover, immunoblotting of cerebellar samples showed that the protein levels of all three *HPI* protein isoforms (*HPI*  $\alpha$ ,  $\beta$  and  $\gamma$ ) were severely reduced in KO animals compared to controls (Supplementary Fig. 1d). The repressive histone H1 also displayed aberrant staining and was more diffuse in *BRCA1* mutant cortices (Supplementary Fig. 2a). Other previously characterized repressive histones, such as Macro H2A1 and heterochromatin markers, such as histone H3 dimethyl Lys 9, did not appear to be affected by *BRCA1* KO (Supplementary Fig. 2b). Since we have previously shown that the *BRCA1* protein preferentially mono-ubiquitinates histone H2A in vitro through its N-terminal RING finger<sup>8</sup>, we investigated whether *BRCA1* is involved in heterochromatin formation through histone H2A ubiquitylation. We found that the staining of mono-ubiquitylated histone H2A, which has recently been implicated in *polycomb*-mediated silencing<sup>9</sup>, corresponded to the heterochromatic centers described in Fig. 1b and that the staining signal was reduced in *BRCA1* mutant brains (Fig. 1c bottom row and Supplementary

Fig.1c right panel). We verified the cell autonomous nature of our observations using cultured embryonic neural progenitor cells (NPCs) in vitro. Ten days after neuronal differentiation, the *BRCA1*-deficient cells displayed a significant reduction in the number of heterochromatic centers (7.85 foci/cell vs 5.00 foci/cell, Student's *t*-test,  $p < 0.0005$ ) (Supplementary Fig. 2c). Taken together, these data strongly suggest that cells lacking *BRCA1* are impaired in the organization of heterochromatin structure.

## Heterochromatic silencing disruption

To ascertain that the observed heterochromatic structural deficiencies are of functional importance, we investigated the transcriptional status of known silenced genes. Defects are expected to result in derepression of normally silenced genes in heterochromatic regions<sup>10</sup>. The murine pericentric heterochromatin consists of stretches of highly repetitive DNA elements, including the major and minor satellite repeats, which make up ~3% and ~0.45% of the mouse genome respectively<sup>11</sup>. Quantitative RT-PCR experiments showed that the levels of minor and major satellite transcripts were elevated 10.2-fold and 27.3-fold, respectively, in the *BRCA1* mutant cortices, indicating a loss of repression in constitutive heterochromatin (Fig. 2a). Other interspersed repetitive elements such as IAP1, SINE1 and the L1 LINE element were largely unaffected. This effect was cell autonomous as it was observed in vitro in cultured embryonic NPCs (Supplementary Fig. 3a). The defects in heterochromatic repression in *BRCA1*-deficient mouse cells could be reversed by re-expression of human *BRCA1* using a lentiviral vector (Fig. 2b). Heterochromatin repression by *BRCA1* is not unique to neural cells because deletion of *BRCA1* in fibroblasts from P7 mouse ribcages also showed upregulation of both satellite transcripts (Supplementary Fig. 3b). Since *BRCA1* is a tumor suppressor in breast cancer we next generated mice harboring a mammary gland specific deletion of *BRCA1* and analyzed the integrity of heterochromatin by qRT-PCR (Fig. 2c). The result demonstrated that lack of *BRCA1* in mammary glands from 6-week old virgin females, disrupted heterochromatic satellite DNA silencing as well as imprinted *H19* epigenetic regulation, consistent with our findings in *BRCA1* null brains (Fig. 2a).

The silencing of constitutive heterochromatin by *BRCA1* is also not limited to mouse cells, as satellite transcripts from human HeLa cells were upregulated upon expression of an shRNA targeting *BRCA1* (Supplementary Fig. 4a). Similarly, reconstitution of the human *BRCA1*-deficient cell line, HCC1937, with a retrovirus expressing wildtype *BRCA1* repressed the expression of satellite DNA transcripts about 20-fold (Fig. 2d). To determine whether the N-terminal RING domain of *BRCA1* is important for the heterochromatic silencing function, we reconstituted HCC1937 cells with either a pathogenic *BRCA1* mutant devoid of ubiquitin ligase activity (T37R, known to confer increased cancer risk) or a polymorphic variant (V11A, indistinguishable from wildtype). The T37R mutant *BRCA1* failed to repress satellite transcripts whereas the V11A repressed the satellite transcripts to an extent similar to wildtype *BRCA1* (Fig. 2d). The lack of repressive activity was not due to reduced binding of *BARD1*, the natural heterodimeric partner of *BRCA1*, as both polymorphic and mutant immunoprecipitated *BARD1* similarly (Supplementary Fig. 4b). To further verify the involvement of the E3-ligase activity of *BRCA1*, we assessed the effects of *BARD1* knockdown on satellite DNA transcription since the heterodimerization between

*BRCA1* and *BARD1* is required for the optimal E3 ligase activity<sup>8,12</sup>. Expression of an shRNA against *BARD1* elevated the satellite DNA transcription in the *BRCA1* reconstituted HCC1937 cells but not in the HCC1937 cells where *BRCA1* is absent and the satellite repeat transcription is already derepressed (Supplementary Fig. 4c). These results from cultured human cells are consistent with data obtained from the *BRCA1* deficient mouse brains, fibroblasts and mammary glands in which satellite DNA transcript induction was observed. Moreover, it appears that ubiquitin E3 ligase activity of *BRCA1* is essential for its role in gene silencing in constitutive heterochromatin.

## ***BRCA1* and heterochromatin relationship**

To gain a mechanistic insight into the role of *BRCA1* in maintaining heterochromatin structure, we first investigated if *BRCA1* directly associates with the constitutive heterochromatic region in vivo. Chromatin immunoprecipitation (ChIP) experiments showed that *BRCA1* was indeed enriched on major and minor satellite DNA (Fig. 2e) in mouse cells and on various  $\alpha$  satellite sequences in HeLa cells (Supplementary Fig. 4d).

We next tested whether *BRCA1* influenced histone ubiquitylation in vivo. The results of ChIP experiments showed that ubiquitylated histone H2A was enriched in both major and minor satellite regions when compared with a histone H3 antibody (Fig. 2f). However, this satellite region enrichment was abrogated in the absence of *BRCA1*, indicating that *BRCA1* integrity is essential for the accumulation of ubiquityl-histone H2A at satellite repeats in vivo. Our data also show that ubiquityl-histone H2A is enriched at the satellite regions of the mouse genome to a greater extent than at other interspersed repeats such as LINE1 (data not shown) suggestive of a distinct heterochromatin. If *BRCA1* is indeed the ubiquitin ligase for pericentric heterochromatic histone H2A, it would imply that the integrity of the RING finger is required for the accumulation of ubiquitylated histone H2A at satellite repeats, as we have shown that the same region is critical for transcriptional silencing in the heterochromatin (Fig. 2d). To test this hypothesis, ChIP experiments were performed using HCC1937 cells reconstituted with *BRCA1*-T37R, *BRCA1*-V11A, or *GFP* (Fig. 2g). The T37R mutant *BRCA1* failed to significantly enrich ubiquitylated histone H2A at satellite regions, in a manner similar to *GFP* control, while the polymorphic *BRCA1*-V11A showed enrichment of ubiquitylated histone H2A within the same regions. A distinct mutant I26A which has been reported to not destabilize the *BARD1* interaction but has no ubiquityl ligase activity<sup>13</sup> fails to complement the loss of *BRCA1* (Supplementary Fig. 5). The ubiquityl ligase function of *BRCA1* appears to be specific as deletion of *Ring1B* or knockdown of *RNF8* fail to appreciably derepress satellite transcription (Supplementary Fig. 6). These findings provide further evidence that the *BRCA1* ubiquitin ligase activity is essential for the maintenance of the ubiquityl histone H2A mark within constitutive heterochromatic regions not only in mouse tissue but also human cells derived from breast cancers.

## **Ubiquitylated histone H2A rescues *BRCA1***

To assess the contribution of ubiquityl-histone H2A to the function of *BRCA1* in heterochromatic silencing we generated a lentiviral vector construct expressing an H2A-Ub fusion protein that mimics the natural ubiquityl histone H2A (Fig. 3a). This was

accomplished by fusing a single ubiquitin moiety to the C-terminus of histone H2A in lieu of the modification of lysine 119. Subnuclear fractionation of cells expressing this fusion protein shows that the H2A-Ub protein and mutants can be incorporated into cellular chromatin (Supplementary Fig. 7). *BRCA1* deficient HCC1937 cells were transduced with lentivirus expressing the H2A-Ub fusion protein. Quantification of the levels of satellite DNA transcription showed that the H2A-Ub expression can re-establish silencing of the satellite repeats (mcBox, SATa and SATIII) within heterochromatin in *BRCA1*-deficient human cells, suggesting that mono-ubiquityl-H2A is likely to be the main target of *BRCA1* ubiquitin E3 ligase that mediates silencing at constitutive heterochromatin (Fig. 3b). Ectopic expression of H2A-Ub (Supplementary Fig. 8) in murine NPCs restored the levels of satellite DNA silencing to more than 90% of endogenous wildtype silencing (Fig. 3c). Mutations within the ubiquitin domain of the fusion protein reduced the ability to restore silencing of the H2A-Ub fusion protein (Supplementary Fig. 9a). H2A-Ub expression in wild type cells had no significant effect on the satellite DNA transcription. To inquire whether the ubiquitylation of histone H2A affected previously well-documented *BRCA1* functions, we tested if the restored satellite DNA silencing in *BRCA1* null cells had any of the expected consequences. It has been reported that primary *BRCA1* null cells display a severe growth defect and *p53*-activated apoptosis and that *p53* inactivation largely rescues the growth defect<sup>14</sup>. By performing BrdU incorporation assays, we found that ectopic expression of H2A-Ub led to a restoration of over 75% of proliferation in *BRCA1* deficient cells, only slightly lower than a *p53* knockdown (Fig. 3d). Again, in this case expression of H2A-Ub bypassed the requirement for *BRCA1*. It is worthy to note that although *p53* is able to rescue the proliferative defect of *BRCA1* deficient cells, it does not rescue the formation of heterochromatic centers, or the derepression of satellite transcripts (Supplementary Fig. 10). We also evaluated the ability of H2A-Ub to suppress apoptosis. Staining for an apoptotic marker, active *caspase3*, showed a similar result (Fig. 3e) to what was seen for BrdU incorporation or transcriptional silencing. Thus H2A-Ub can suppress the apoptosis induced by the loss of *BRCA1*. Taken together, ectopic expression of H2A-Ub not only restored silencing but also rescued the proliferative defect and apoptosis of *BRCA1* deficient cells. This rescue of the proliferative capacity of H2A-Ub is specific for the ubiquitylation modification, as mutations within the ubiquitin domain can abrogate the effect (Supplementary Figs. 9b and 9c). Homologous recombination, another reported cellular process in which *BRCA1* has been implicated, can also be rescued by ectopic H2A-Ub expression in the absence of *BRCA1*, whereas the same protein with a triple point mutation within the ubiquitin domain is unable to complement (Supplementary Fig. 11). Thus, mono-ubiquityl-H2A is likely the principal target of *BRCA1* for maintaining heterochromatin integrity in vivo.

## Heterochromatin and *BRCA1* tumor suppression

We next investigated the significance of heterochromatin function in *BRCA1* mediated tumor suppression. Mouse breast tumors (Fig. 4a) were harvested from animals where *BRCA1* was deleted in the mammary gland (also shown in Fig. 2c). Analyses by qRT-PCR showed disrupted satellite DNA silencing in *BRCA1* deficient mouse mammary tumors, but not in tissues where *BRCA1* is intact (Fig. 4b). Furthermore, we analyzed satellite transcripts

in human breast tumors derived from eight individual *BRCA1* mutation carriers and found significant derepression of the expression of alpha satellite repeats: CFXr and SATa (two of human alpha satellite DNA sequences; Fig. 4c). The differences are apparent, even without normalizing for any other factors (e.g. the percentage of non-tumor cell contamination), in comparison with a cohort of normal breast biopsies.

### Satellite transcript induced genomic instability

To investigate whether the observed satellite DNA derepression in mouse and human breast cancers is either an epiphenomenon or of etiological significance for the genesis of cancer, we ectopically expressed satellite DNA from a transduced lentiviral vector (Fig. 5a) in cultured primary human mammary epithelial cells (HMECs). These cells increased abnormal mitotic figures, including bridged and lagging chromosomes and disorganized metaphases shown by DAPI staining (Figs. 5b and supplementary Fig.12).

Immunofluorescence staining of pericentrin and tubulin revealed amplified centrosomes, as previously described in *BRCA1* null cells (Fig. 5c). Cells overexpressing satellite RNA also displayed numerous foci of  $\gamma$ H2Ax (Fig. 5d), a well-established marker of DNA double strand breaks. This mirrors the phenotype of *BRCA1* deficient cells. Flow cytometric analyses showed an impaired mitotic spindle checkpoint when cells overexpressing satellite DNA were treated with nocodazole (Fig. 5e), which has also been reported as a *BRCA1* loss phenotype<sup>15</sup>. Just as expected, ectopic expression of satellite RNA was able to create a deficiency in homologous recombination similar in magnitude to the knockdown of *BRCA1* (Supplementary Fig.13). Importantly, the cellular defects associated with satellite RNA overexpression are generally considered leading causes for genomic instability.

### Discussion

The present work shows that *BRCA1* deficiency impairs the integrity of constitutive heterochromatin which leads to the disruption of gene silencing at the tandemly repeated DNA regions, probably through the loss of ubiquitination of histone H2A. *BRCA1/BARD1* proteins preferentially mono-ubiquitylate H2A at high stoichiometry<sup>8</sup>, which comprises 5-15% of the total cellular nucleosomal histone H2A<sup>16</sup>. *BRCA1* accumulates H2A-Ub at satellite DNA regions, which encompass ~3.45% of the mouse genome<sup>11</sup> (Fig. 2f) and itself localizes there by ChIP experiments (Fig. 2e). The *BRCA1* RING domain is essential for both satellite DNA binding and silencing. *BARD1* is also required for repression of satellite repeats and loss of *BARD1* is of no consequence if *BRCA1* is already absent (Supplementary Fig. 4c). Although the exact function is not well understood, the emerging notion is that mono-ubiquitinated histone H2A is most frequently associated with transcriptionally repressive states<sup>9,17,18</sup>. Other ubiquityl ligases: *Ring1A/B*, of the polycomb repressive complex 1 (PRC1), and *RNF8*, a DNA repair ligase<sup>19,20</sup> ubiquitylate H2A but do not contribute to the silencing of satellite repeats (Supplementary Fig. 6). Similarly, *BAP1*, a known *BRCA1* binding protein, has been shown to antagonize the polycomb complex by deubiquitinating histone H2A<sup>21,22</sup>. Since a mimic of natural ubiquityl-H2A can rescue the most prominent *BRCA1* deficiency defects, it suggests that ubiquitylation of histone H2A is likely to be the primary substrate of *BRCA1* (Figs. 3 and Supplementary Fig. 9).



It is worth noting that overexpression of the satellite transcripts in the presence of wildtype *BRCA1* partially phenocopies *BRCA1* loss (Fig. 5), including centrosome amplification and  $\gamma$ H2Ax foci formation. These observations suggest constitutive heterochromatin formation prevents DNA damage or a DNA damage response. The function of satellite transcripts is largely unknown. However, it has been known that the integrity of centromeric heterochromatin requires an unknown RNA component<sup>7</sup> that could conceivably be satellite transcripts.

Tumor suppression by *BRCA1* was presumed to occur via homologous recombination<sup>3</sup>. Recent work showed that cells expressing a mutant *BRCA1* devoid of E3 ligase activity do not exhibit defects in homologous recombination<sup>13</sup>. Thus, the defect in genomic instability in *BRCA1* null cells seems not to require this function although an intact RING domain is required for recovery from gamma irradiation<sup>23</sup>. Nevertheless, mutations that compromise the ubiquityl ligase activity of *BRCA1* are cancer predisposing and constitute hotspots of mutational occurrences in patients<sup>23</sup>. Our work shows that satellite DNA repression indeed requires an intact *BRCA1* RING domain, consistent with satellite DNA derepression observed in *BRCA1* tumors (Fig. 2d). Indeed satellite transcripts in clinical *BRCA1* patient tumors are upregulated (Fig. 4c). Since satellite transcripts when expressed ectopically can induce centrosome amplification, cell cycle check point defect and the DNA damage response, it fulfills the criteria to be a main effector of *BRCA1* mediated tumorigenesis. Although it is presently unclear how these processes are elicited at a mechanistic level it is tempting to speculate that histone H2A ubiquitylation pathways are involved. Alternatively, the derepression of satellite might be elicited by some indirect “global effect” *in trans* that would not require H2A-Ub to be at the derepressed site. It is conceivable that induction of satellite transcripts could be developed into a marker of loss of heterozygosity for clinical diagnostic application. Recent work has shown that a variety of epithelial cancers overexpress satellite repeats that encompass up to 50% of cellular transcripts<sup>24</sup>. Although the pathological significance of this finding is unknown, our work suggests that these could contribute to the evolution of the cancer cell through the induction of genomic instability (Supplementary Fig. 14).

## Methods Summary

Cell culture, virus infection, quantitative RT-PCR and ChIP experiments. Primary neural progenitor cells were isolated from adult mice containing one *BRCA1* null allele and one floxed *BRCA1* allele as described<sup>25</sup>. Embryonic neural progenitors were isolated and cultured as described<sup>26</sup>. Cultured cells were infected with retroviruses with an MOI of 5 for 12-h and harvested at the time indicated in corresponding figure legends. Cells were harvested with TRIZOL for RNA extraction. Reverse transcription was carried out using SuperScript III First-strand Synthesis System (Invitrogen). The quantitation of PCR products was analyzed with SYBR Green using ABI PRISM 7700 Sequence Detection system software (Applied Biosystems). Crosslinking and ChIP were performed as a modified version of a protocol described by the Clevers laboratory<sup>27</sup> and supplementary information.

Full Methods and any associated references are available in the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

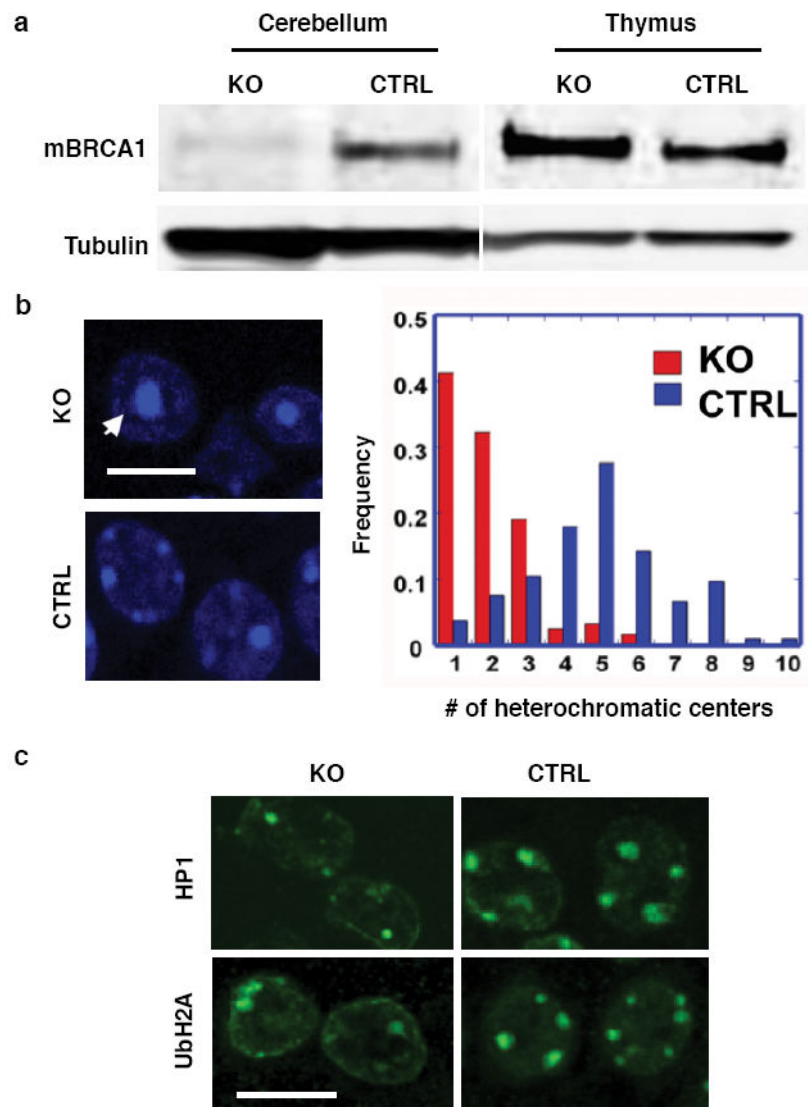
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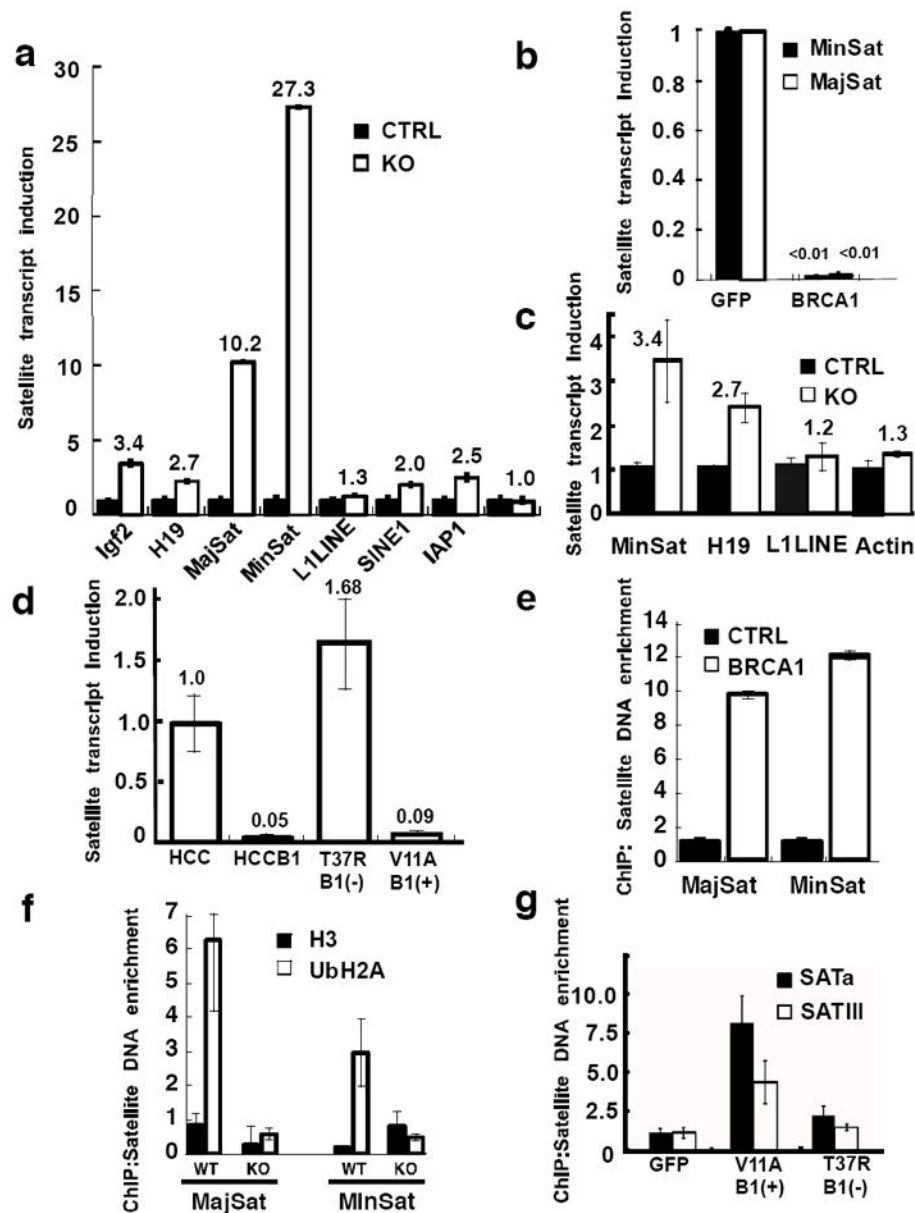


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**Figure 1. BRCA1 deficiency impairs heterochromatin structure**

**a**, Expression of BRCA1 is diminished in the *Nestin-Cre BRCA1* knockout brains as shown by immunoblotting. KO: samples from a P7 *BRCA1* KO (*Brca11*<sup>+/-</sup>;*Brca5-13cK*<sup>+/-</sup>;*nestin-Cre*<sup>+</sup>) mouse. CTRL: samples from a control P7 (*Brca11*<sup>+/-</sup>;*Brca5-13cK*<sup>+/-</sup>;*nestin-Cre*<sup>-</sup>). BRCA1 immunoblot (upper panel), tubulin loading control (lower panel). **b**, Lack of BRCA1 induces changes in the nuclear morphology of P7 KO cortical cells. The numbers of strong DAPI staining nuclear foci/cell were counted and their frequencies plotted (right panel). **c**, Confocal microscopic images of brain sections from BRCA1 conditional KO and control mice (P7) stained with antibodies against *HP1* and ubiquitin-H2A (UbH2A).



**Figure 2. BRCA1 and its ubiquitin E3 ligase activity are required for gene silencing in constitutive heterochromatin**

**a**, Quantitative RT-PCR showed that heterochromatic regions or certain imprinted genes (*Igf2* & *H19* but not *Igf2R*) were upregulated in *BRCA1* KO brains. Internal controls: *cyclophilin* or *18SRNA*. **b**, Rescue of the *BRCA1* mediated repression in NPCs by exogenous human *BRCA1* but not *GFP* after infection with retroviral *CRE-GFP*. **c**, Quantitative RT-PCR experiments showed that heterochromatic regions or imprinted genes were upregulated in *BRCA1* KO mammary glands (6-week virgin). **d**, The ubiquitin E3 ligase activity of *BRCA1* is required for heterochromatin silencing. HCC1937 cells were reconstituted with either wild type *BRCA1* (HCCB1) or *BRCA1* with a mutation in the RING domain, T37R<sup>23</sup>. P7 cerebellar ChIP experiments with antibodies against (e) mouse *BRCA1* or *VSVG* protein

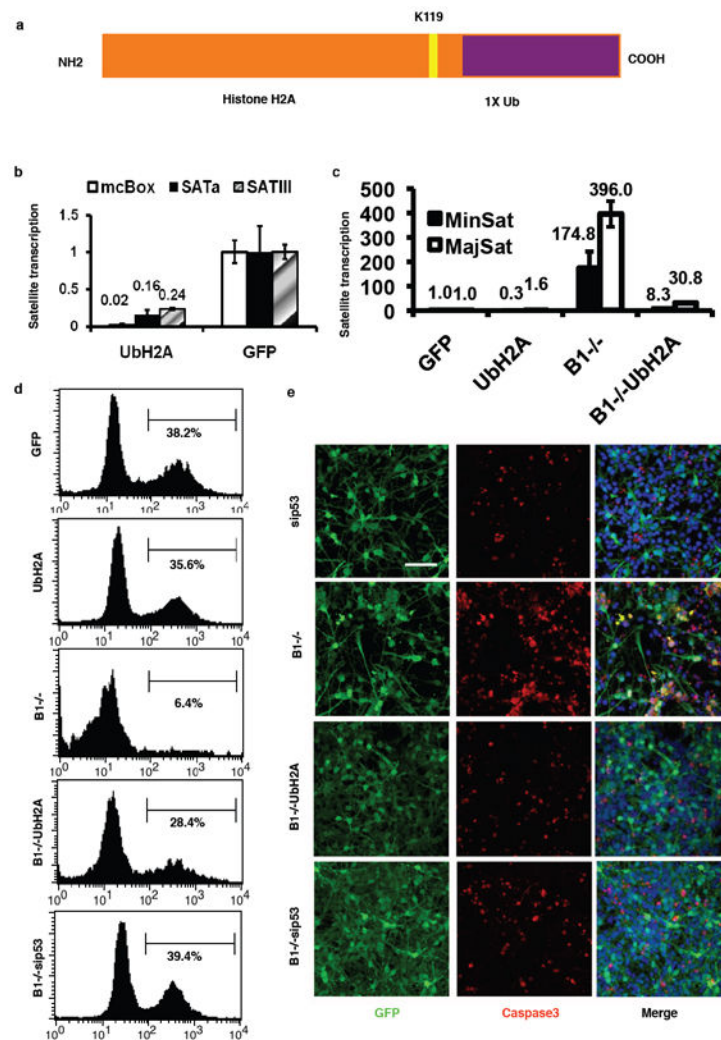
as a control and (f) ubiquityl-histone H2A or histone H3 protein as a control. Enrichment of major and minor satellite DNA was measured by quantitative PCR relative to 18S control. g, ChIP experiments were performed using ubiquitinated histone H2A antibody as in (f) and HCC1937 cells reconstituted with *GFP*, *BRCA1* (V11A) or a mutant (T37R). Error bars are shown as s.d. Each result shown is representative of three independent experiments.

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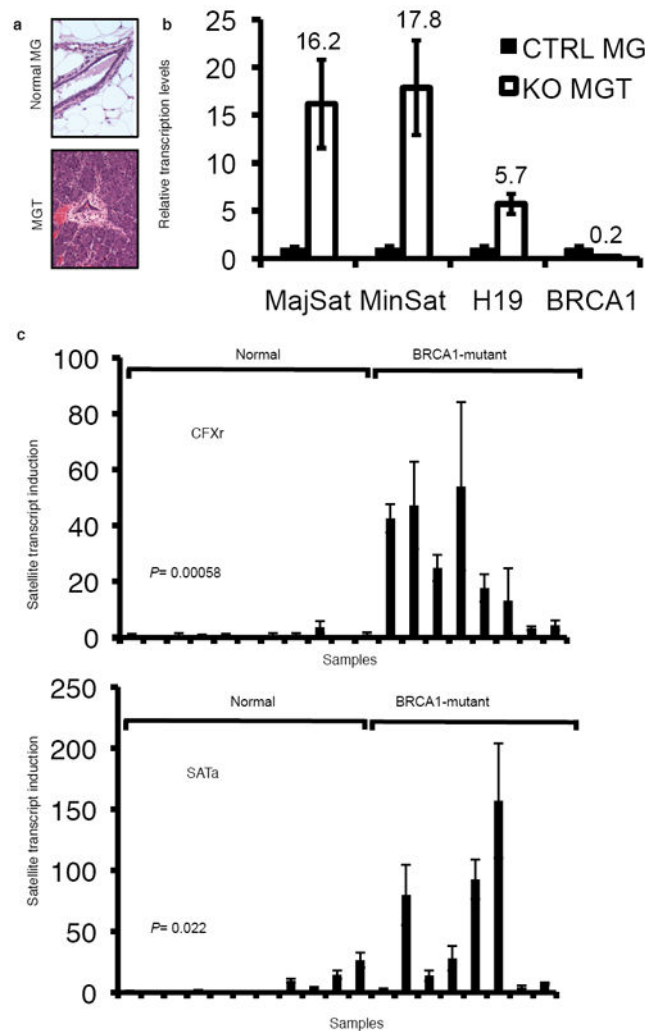
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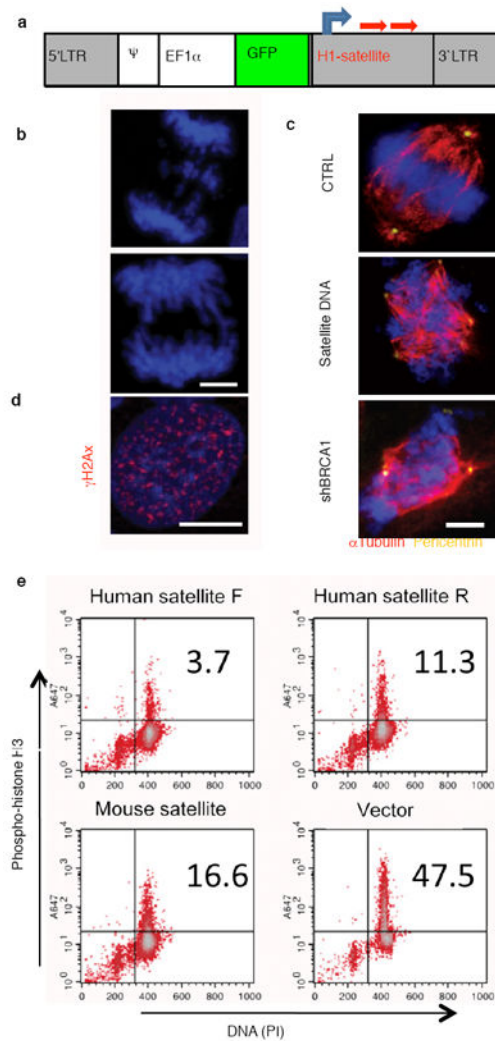
**Figure 3. Ubiquitinated histone H2A is directly involved in BRCA1 mediated heterochromatic silencing**

**a**, A diagram showing the artificial ubiquitin-H2A expression vector. **b**, HCC1937 cells were infected with a lentiviral vector expressing ubiquitylated histone H2A mimic fusion protein or *GFP* as a control and the levels of alpha satellite variants (mcBox, SATa and SATIII) transcription were measured by qRT-PCR. **c**, Ectopic expression of ubiquitylated histone H2A re-established heterochromatin silencing in *BRCA1* null NPCs. NPCs were infected with *GFP* alone, *ubH2A*, *CRE-GFP* alone and *CRE-GFP* coinfecting with *UbH2A*. The levels of satellite DNA transcription were measured by qRT-PCR. **d**, NPCs showed increased proliferation upon expression of ubiquitylated histone H2A in *BRCA1* null NPCs. Two days after infection (2 dpi), cells were labeled with BrdU prior to fixation, immunostaining and FACS analysis. Result shown is representative of 3 independent experiments. **e**, NPCs showed reduced apoptosis upon expression of ubiquitylated histone H2A in *BRCA1* null NPCs. NPCs were cultured and infected as in (**d**). Cells were fixed, immunostained with anti-activated *caspase 3* antibody and subjected to confocal imaging. Error bars are shown as s.d. Scale bar: 100  $\mu$ m



**Figure 4. Derepression of satellite DNA transcription occurs in BRCA1-deficient breast cancers**  
**a**, Haematoxylin/eosin staining of *BRCA1*KO mouse breast cancer. Because *BRCA1* KO female mice develop mammary tumors very rarely, these mice were crossed into a p53 heterozygous background for accelerated tumorigenesis. MG, mammary gland from normal mouse littermates. MGT, mammary tumors from *BRCA1*KO mouse (*Brca1*<sup>+/-</sup>;*Brca5-13cK*<sup>+/-</sup>;*MMTV-Cre*<sup>+</sup>;*p53*<sup>+/-</sup>) developed at 6 months. **b**, Quantitative RT-PCR experiments showed that heterochromatic regions and imprinted genes were upregulated in mouse *BRCA1* KO breast cancer in comparison to that of wildtype mammary glands from littermates (internal control *18S RNA*). The result shown is an aggregate of 5 mouse independent sample pairs. **c**, Quantitative RT-PCR experiments showed the satellite DNA transcripts, CFXr and SATa, significantly derepressed (Student's 2-tailed *t*-test) in human *BRCA1*-mutant breast tumors (n=8) in comparison with that of normal breast tissues (n=11). Ct values of each sample were normalized with *GAPDH*. Error bars are shown as s.d.





**Figure 5. Ectopic expression of satellite DNA leads to genomic instability in human mammary epithelial cells**

**a**, A diagram of the lenti-viral vector expressing the human or mouse satellite DNA sequence (red arrows) under the H1 promoter (gray arrow). **b**, **c**, **d**, Overexpression of satellite RNA induced mitotic catastrophe (**b**), centrosome amplification (**c**), histone  $\gamma$ H2Ax phosphorylation (**d**), in primary HMECs. **e**, Defective mitotic checkpoints induced by satellite DNA overexpression. 48h after transduction of a lentivirus expressing human satellite DNA sequence with forward direction (human satellite F), or with reverse direction (human satellite R), or mouse satellite DNA sequence, or an empty vector, U-2OS cells were blocked with thymidine, followed by treatment of nocodazole. The cells were stained with phospho-histone H3 antibody and PI prior to flow cytometric analysis. The shown experiment is representative of 3 independent replicates.