

Association of BRCA1 with the inactive X chromosome and XIST RNA

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Breast cancer, early onset 1 (BRCA1) encodes a nuclear protein that participates in breast and ovarian cancer suppression. The molecular basis for the gender and tissue specificity of the BRCA1 cancer syndrome is unknown. Recently, we observed that a fraction of BRCA1 in female cells is localized on the inactive X chromosome (Xi). Chromatin immunoprecipitation (ChIP) experiments have demonstrated that BRCA1 physically associates with Xi-specific transcript (XIST) RNA, a non-coding RNA known to coat Xi and to participate in the initiation of its inactivation during early embryogenesis. Cells lacking wild-type BRCA1 show abnormalities in Xi, including lack of proper XIST RNA localization. Reintroduction of wild-type, but not mutant, BRCA1 can correct this defect in XIST localization in these cells. Depletion of BRCA1 in female diploid cells led to a defect in proper XIST localization on Xi and in the development of normal Xi heterochromatic superstructure. Moreover, depletion of BRCA1 led to an increased likelihood of re-expression of a green fluorescent protein (GFP) reporter gene embedded on Xi. Taken together, these findings are consistent with a model in which BRCA1 function contributes to the maintenance of proper Xi heterochromatin superstructure. Although the data imply a novel gender-specific consequence of BRCA1 loss, the relevance of the BRCA1/Xi function to the tumour suppressor activity of BRCA1 remains unclear and needs to be tested.

Keywords: BRCA1; XIST; X chromosome inactivation; heterochromatin; breast cancer

1. INTRODUCTION

The *BRCA1* gene encodes an 1863 residue nuclear protein that has little homology to other known proteins. Women carrying a germline mutation in *BRCA1* experience a greatly increased lifetime risk of breast and ovarian cancer. The tumours that arise in these individuals all reveal loss of the wild-type and retention of the mutant allele of *BRCA1*, implying that this gene functions as a tumour suppressor. Interestingly, men who harbour a *BRCA1* mutation do not have a clear increase in cancer predisposition (Thompson & Easton 2002).

BRCA1 is a nuclear protein found in all proliferating cells that have been tested and whose expression appears to be cell-cycle regulated. Extensive investigation has shown that *BRCA1* probably plays a role in several fundamental cellular processes including the maintenance of genomic integrity, transcriptional regulation and cell-cycle checkpoint regulation (Deng & Brodie 2000; Scully & Livingston 2000; Venkitaraman 2002). Cells lacking wild-type BRCA1 rapidly develop chromosomal abnormalities and experience abnormal DNA damage repair and altered cell-cycle checkpoint regulation. All of these defects can be partly complemented by reintroduction of wild-type

BRCA1 (Gowen *et al.* 1998; Moynahan *et al.* 1999; Scully *et al.* 1999).

Overexpression of BRCA1 can alter expression of a variety of genes including GADD45 (growth arrest and DNA damage-inducible 45) and may modulate ligand and ligand-independent activation of the oestrogen receptor (Harkin *et al.* 1999; Zheng *et al.* 2000; Fan *et al.* 2001). Recent evidence suggests that BRCA1 may have a role in the regulation of chromatin structure, as seen by its association with the SWI/SNF (mating type switch/sucrose non fermenting) complex and by reports that overexpression of BRCA1 can alter the structure of certain chromatin domains (Bochar *et al.* 2000; Ye *et al.* 2001). BRCA1, together with its partner protein, BARD1, also exhibits *in vitro* E3 ubiquitin ligase activity (Hashizume *et al.* 2001). How these potential functions are related to BRCA1-tumour suppression, and how they contribute to the gender and tissue specificity of the BRCA1 cancer syndrome in humans remain unclear.

2. BRCA1 LOCALIZES TO THE INACTIVE X-CHROMOSOME IN SPERMATOCYTES AND FEMALE SOMATIC CELLS

BRCA1 mRNA and protein are highly expressed in the testis. In zygotene spermatocytes, BRCA1 is detected on the unsynapsed arms of developing synaptonemal complexes together with RAD51 (Scully *et al.* 1997). In the more abundant pachytene spermatocytes, BRCA1 heavily

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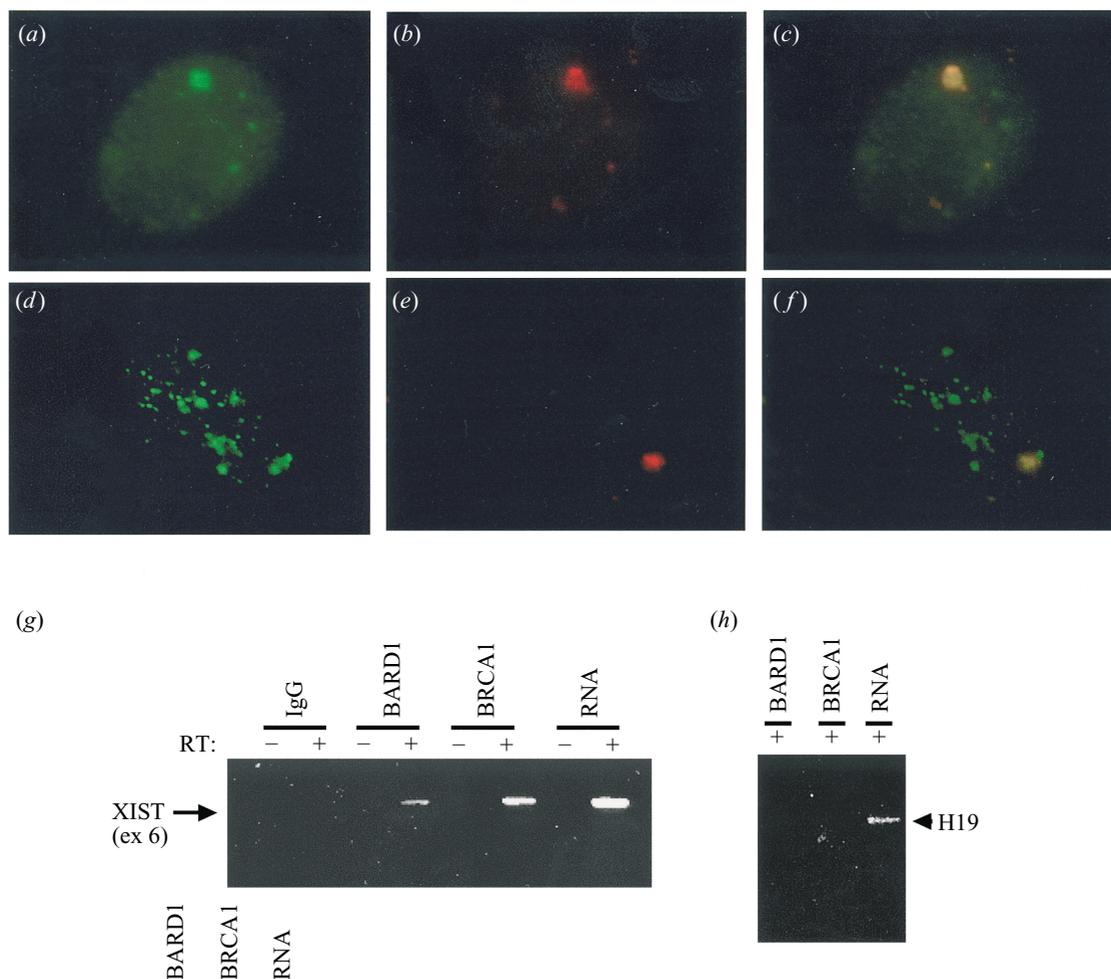


Figure 1. Association of BRCA1 and markers of Xi (reproduced from Ganesan *et al.* (2002)¹ with permission). (a–f) Combined immunostaining of telomerase immortalized human mammary epithelial cells (a–c) and WI-38 female human fibroblasts (d–f) with a monoclonal antibody to BRCA1 (a,d), and RNA FISH for XIST (b,e). Merged images are shown in the right panels (c,f). (g) ChIP was performed on 293 cells by using purified antibodies to BRCA1, BARD1 and an irrelevant antibody. RNA was extracted from each ChIP and subjected to RT-PCR using primers specific for a region in exon 6 of XIST. The (+) and (–) signify PCR amplification with and without first round RT. The last two lanes are controls, using cellular RNA as a template. (h) ChIPs, performed on 293 cell extract, were subjected to RT-PCR using primers specific for H19 RNA.

decorates the unpaired X chromosome (Scully *et al.* 1997; Ganesan *et al.* 2002). In pachytene cells, the unpaired X and Y chromosomes undergo dramatic changes in chromatin structure, becoming densely heterochromatic, transcriptionally silenced, and localized at the nuclear periphery in a discrete structure called the XY body (Handel & Hunt 1992). The pachytene cell X chromosome accumulates the non-coding XIST RNA, becomes enriched with the histone variant MH2A1, and undergoes methylation of histone H3 on lysine 9 (Richler *et al.* 1992, 2000; Ayoub *et al.* 1997; Costanzi & Pehrson 1998). These changes are remarkably similar to the changes that occur on Xi in female somatic cells (Clemson *et al.* 1996; Jaenisch *et al.* 1998; Avner & Heard 2001; Huynh & Lee 2001), and led us to investigate whether BRCA1 functionally interacts with Xi.

BRCA1 immunostaining appears in proliferating cells as multiple nuclear foci that are most apparent during S and G2 phases of the cell cycle. In a variety of human female (but not male) cell lines and strains, a subset of unsynchronized cells demonstrated colocalization of

BRCA1 with markers of Xi, such as MH2A1 and XIST RNA on a nuclear structure (figure 1). This colocalization was apparent in only a small fraction of cells, but it was present in multiple female cell lines and strains and appears to be cell-cycle dependent (Ganesan *et al.* 2002).

3. ASSOCIATION OF BRCA1 AND BARD1 WITH XIST RNA

ChIP performed with antibodies specific to BRCA1 demonstrated the presence of XIST RNA (figure 1). No such band was detected when irrelevant antibody was used. Moreover, ChIP, performed with an antibody to BARD1, a structurally related protein that efficiently heterodimerizes with BRCA1, also co-immunoprecipitated XIST RNA (figure 1). By contrast, the same ChIPs lacked H19 RNA, another non-coding RNA (Ganesan *et al.* 2002). These findings suggest that BRCA1 and BARD1 interact, directly or indirectly, with XIST RNA.

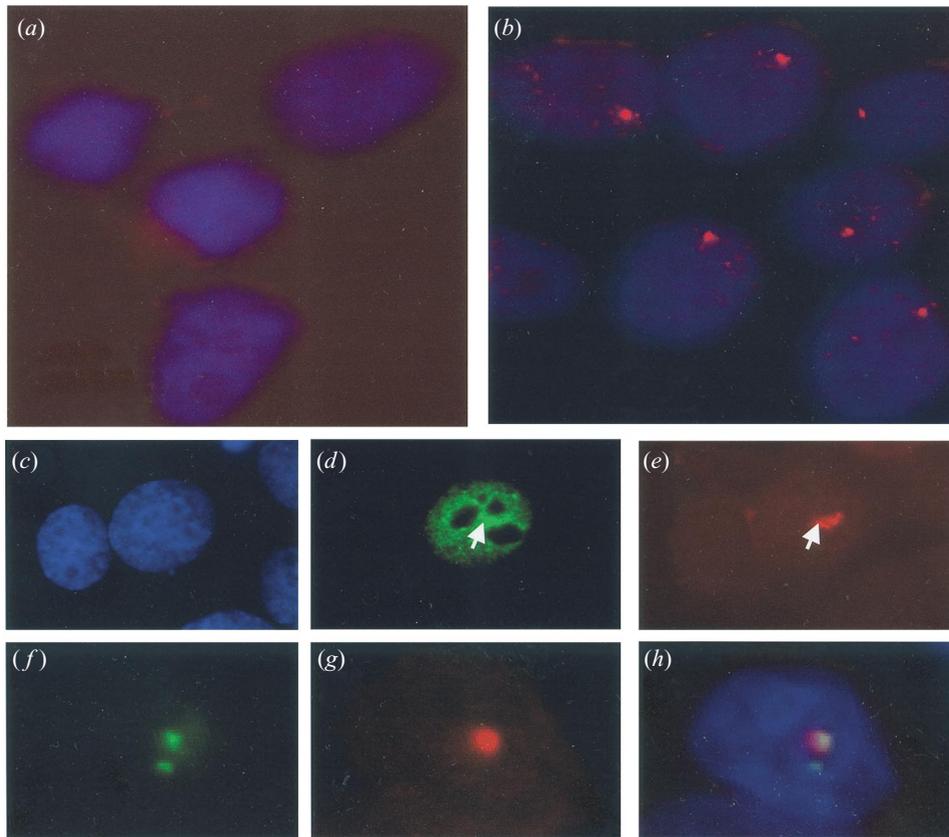


Figure 2. XIST RNA FISH analysis of naive and BRCA1-reconstituted HCC1937 cells (reproduced from Ganesan *et al.* (2002)¹ with permission). (a,b) XIST RNA FISH staining of vector-reconstituted HCC (a) or HCC that were stably reconstituted with wild-type BRCA1 (b). (c–e) HCC stably transfected with a doxycycline inducible wild-type BRCA1 vector (HCC clone 5) were exposed to doxycycline for 24 h and subjected to combined immunostaining for BRCA1 (d) and RNA FISH for XIST (e). Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI) (c). The arrow points to the region of greatest BRCA1 staining intensity, which coincides with the region that stains for XIST RNA. (f–h) Simultaneous DNA FISH for an X-chromosome probe (f) and RNA FISH for XIST (g) were performed on HCC clone 5 cells stimulated with doxycyclin. A merged image is shown in (h).

4. BRCA1-DEFICIENT TUMOUR CELLS REVEAL ABNORMALITIES IN XIST LOCALIZATION

To determine whether BRCA1 maintains a functional interaction with Xi, certain characteristic features of Xi were examined in tumour cells lacking wild-type BRCA1. HCC1937 cells are human breast cancer cells that lack wild-type BRCA1. Most HCC1937 cells contain two X-chromosomes, although the line is aneuploid, and certain cells contain greater than two X chromosomes (Ganesan *et al.* 2002). Despite having at least two X-chromosomes, this cell line lacks several normal features of Xi, including focal accumulation of XIST and focal staining with antibody to MH2A1 (figure 2; Ganesan *et al.* 2002). Similar results were obtained both in frozen section of breast and ovarian tumours arising in women with germline BRCA1 mutations, suggesting that loss of focal XIST and MH2A1 localization on Xi is a common consequence of BRCA1 loss. Those sporadic tumours that were examined and expressed wild-type BRCA1 revealed normal localization of both XIST and MH2A1.

When a wild-type BRCA1 allele was reintroduced by retroviral infection into HCC1937 cells and expressed at near physiological levels, or when we triggered stable over-expression of an inducible plasmid BRCA1 vector, most of the BRCA1 reconstituted HCC1937 cells revealed a

single focus of XIST staining by RNA FISH (figure 2). Similar reconstitution with disease-associated, point missense-mutants of BRCA1 failed to induce focal XIST staining, suggesting that intact BRCA1 is required for this effect. Naive and wild-type BRCA1-reconstituted HCC1937 cells, although characterized by clear differences in XIST staining by RNA FISH, did not reveal measurable differences in XIST RNA levels as seen by quantitative RT-PCR. This observation suggests that BRCA1 does not affect XIST RNA synthesis or stability, but, by exclusion, may support proper localization of XIST RNA on Xi.

5. SUPPRESSION OF BRCA1 SYNTHESIS DISRUPTS XIST RNA LOCALIZATION

When small interfering RNAs specific for BRCA1 were used to acutely suppress synthesis of BRCA1 in female diploid fibroblasts, focal XIST staining was lost in a significant proportion of these cells (figure 3). Similar suppression of BRCA1 synthesis also led to a loss of focal MH2A1 staining in these cells (Ganesan *et al.* 2002). These observations suggest that acute loss of BRCA1 function disrupts the normal localization of both XIST RNA and MH2A1 on Xi.

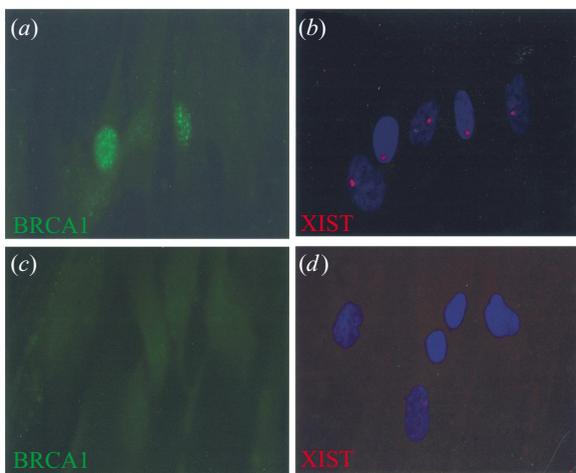


Figure 3. Effect of BRCA1 RNAi on XIST/Xi staining (reproduced from Ganesan *et al.* (2002)¹ with permission). WI-38 were transfected with control siRNA (*a,b*) or with BRCA1-specific siRNA (*c,d*). After 72 h, all cells were analysed by immunostaining for BRCA1 (*a,c*) and by FISH for XIST RNA (*b,d*).

6. STATE OF X CHROMOSOME GENE EXPRESSION IN BRCA1-DEPLETED CELLS

To determine whether acute suppression of BRCA1 synthesis leads to a detectable change in Xi gene expression, siRNA-mediated suppression of BRCA1 was performed in a reporter cell line. This cell line is derived from female murine fibroblasts in which one X-chromosome carries a non-functioning *Xist* allele and the other X expresses a wild-type *Xist* allele and carries a GFP transgene (similar to cells analysed by Csankovszki *et al.* (2001)). The X chromosome bearing the GFP transgene is, thus, normally silenced. As assessed by fluorescent cell sorting, exposure of these cells to AZC, a potent inhibitor of DNA methylation, led to reactivation of the GFP allele in a fraction of cells in this and related cell lines (Csankovszki *et al.* 2001; figure 4). Treatment of these cells with siRNA specific for BRCA1 also led to the appearance of a population of GFP-expressing cells (figure 4). The relative increase in GFP-expressing cells over background, both after AZC or BRCA1 RNAi treatment, although clearly incomplete, was similar in magnitude to the effect observed when the *Xist* gene was somatically deleted from an analogous murine cell line (Csankovszki *et al.* 2001). These data suggest that loss of BRCA1 may lead to a measurable destabilization of the maintenance of silencing on Xi.

7. SUMMARY

The data summarized above demonstrate that loss of BRCA1 can lead to a specific disruption of several characteristic features of Xi, including the normal localization of XIST RNA. The association between loss of BRCA1 expression and failure of proper XIST localization was observed not only in cultured cells but also in multiple BRCA1 $-/-$ primary human tumour cells. These results imply that loss of XIST localization is a naturally occurring event in certain BRCA1-deficient tumour cells. Acute suppression of BRCA1 synthesis led an increased

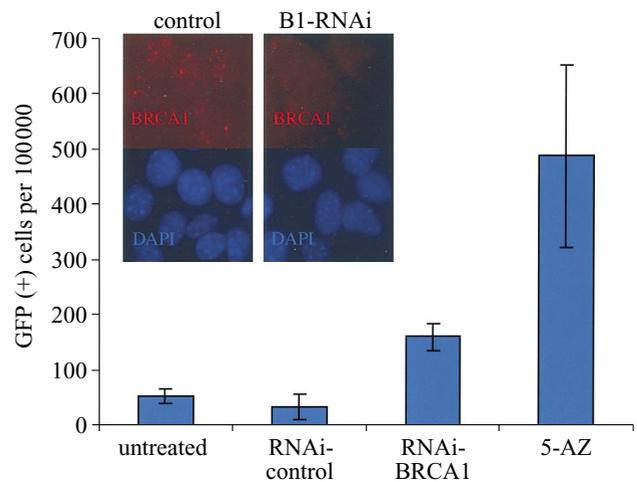


Figure 4. Effect of BRCA1 RNAi on expression of an Xi GFP transgene (reproduced from Ganesan *et al.* (2002)¹ with permission). A murine fibroblast line that carries a non-functional *Xist* allele on one X-chromosome and a GFP transgene on the other was either mock-transfected, transfected with control RNAi, transfected with BRCA1-specific RNAi or exposed to AZC. After 72 h, cells were analysed by fluorescent activated cell sorting (FACS) for GFP expression. The number of cells gated GFP positive per 100 000 cells is plotted for each condition. The inset figures depict the effect of BRCA1-specific RNAi on BRCA1 immunofluorescence in these cells.

incidence of reactivation of a GFP transgene located on Xi. Taken together, these data suggest that intact BRCA1 function may be necessary for proper maintenance of X-chromosome inactivation.

Whether this aspect of BRCA1 function is related to its tumour suppression function is unclear. However, it is tempting to speculate that loss of BRCA1 may, in certain female cells, have the additional effect of leading to heightened expression of some X chromosomal genes that may play a role in the development of breast and ovarian cancer. In this regard, it was recently reported that BRCA1-deficient ovarian cancers displayed overexpression of a set of X-chromosomal genes (Jazaeri *et al.* 2002).

Abundant prior evidence supports a role for abnormal X-chromosome dosage in the development of breast and ovarian cancer. A subset of malignant breast and ovarian tumours lack a detectable Barr body (Kimmel 1957; Moore & Barr 1957; Perry 1972). Sporadic breast cancers composed of cells that contain at least two X chromosomes but a low level of detectable Barr bodies were more aggressive than their Barr body-containing counterparts and tended to have lower oestrogen receptor expression (Rosen *et al.* 1977). Similarly, men with Klinefelter's syndrome (genotype XXY) have a predisposition to both gynecomastia and breast cancer, again suggesting a role for increased X gene dosage in breast growth and carcinogenesis (Swerdlow *et al.* 2001). One of the most common cytogenetic abnormalities observed in male breast cancer is an increase in the number of X chromosomes (Teixeira *et al.* 1998; Rudas *et al.* 2000). Given these observations, it is possible that defective maintenance of X chromosome dosage is a predisposing event to the neoplastic progression of breast and ovarian epithelial cells.

The interaction of BRCA1 with Xi and XIST may be part of a more general role of BRCA1 in the regulation of chromatin structure. Only a fraction of BRCA1 is localized to Xi in female cells. During the S and G2 phases of the cell cycle, it also concentrates on another group of focal, nuclear structures (Scully *et al.* 1996). Because the Xi is a prominent form of facultative heterochromatin, the aforementioned results raise the possibility that these BRCA1 nuclear dots also contain heterochromatic DNA structures. If so, perhaps BRCA1 participates in regulating the structure and function of certain heterochromatic structures during DNA replication and/or repair.

Specific, non-coding RNA molecules participate in regulating sex chromosome dosage compensation through the control of chromatin structure not only in mammals but also in *Drosophila* (Kelley & Kuroda 2000). RNA molecules also appear to support the higher-order structure of peri-centromeric heterochromatin (Maison *et al.* 2002). RNA probably plays a greater role in chromatin structure control than previously considered. Future investigations will determine whether the (direct or indirect) interaction of BRCA1 with XIST RNA is a reflection of a larger role for BRCA1 in influencing aspects of chromatin structure through RNA interactions.

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ENDNOTE

¹Figures 1–4 were adapted and reprinted from Ganesan *et al.* (2002). Copyright (2002), with permission from Elsevier.

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GLOSSARY

- AZC: 5-azacytadine
BARD1: BRCA1-associated ring domain-1
BRCA1: breast cancer, early onset 1
ChIP: chromatin immunoprecipitation
FISH: fluorescent *in situ* hybridization
GFP: green fluorescent protein
MH2A1: macrohistone H2A1
RT: reverse transcriptase
siRNA: small interfering RNA
Xi: inactive X chromosome
XIST: Xi-specific transcript