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DSS1 is required for RAD51 focus formation and genomic stability in mammalian cells

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***BRCA2* is a breast cancer susceptibility gene implicated in the repair of double-strand breaks by homologous recombination with RAD51. BRCA2 associates with a 70-amino-acid protein, DSS1, but the functional significance of this interaction has remained unclear. Recently, deficiency of a DSS1 orthologue in the fungus *Ustilago maydis* has been shown to cause a defect in recombinational DNA repair. Here we have investigated the consequences of DSS1 depletion in mammalian cells. We show that like BRCA2, DSS1 is required for DNA damage-induced RAD51 focus formation and for the maintenance of genomic stability, indicating a function conserved from lower eukaryotes to humans. However, DSS1 seems to be not required for BRCA2 or RAD51 stability or for BRCA2 and RAD51 to interact, raising the possibility that DSS1 may be required for the BRCA2–RAD51 complex to become associated with sites of DNA damage.**

Keywords: DSS1; homologous recombination; breast cancer susceptibility; BRCA2; RAD51; DNA damage

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INTRODUCTION

Mutations in *BRCA2* result in breast cancer susceptibility and have been implicated in Fanconi's anaemia (D'Andrea & Grompe, 2002; Howlett *et al*, 2002; Tutt & Ashworth, 2002; Venkitaraman, 2002). *BRCA2* encodes a large protein of 3418 amino acids, which binds to and regulates the activity of RAD51 in homologous recombination. Following DNA damage that creates double-strand breaks (DSBs), BRCA2 and RAD51 colocalize to foci in the nucleus, a process that is dependent on BRCA2. At the break site, RAD51 catalyses strand exchange between sister chromatids or homologous chromosomes resulting in error-free repair. A defect in this repair pathway leads to elevated spontaneous and damage-induced chromosome aberrations due to the repair of DSBs by error-prone mechanisms such as non-homologous end joining and single-strand annealing (Tutt & Ashworth, 2002; Venkitaraman, 2002).

The carboxy-terminal region of BRCA2 binds to a highly conserved 70-amino-acid protein DSS1 (also known as SHFM1, Shfdg1 and SEM1; Marston *et al*, 1999), which was originally identified as a candidate gene for split hand/split foot syndrome (Crackower *et al*, 1996). The crystal structure of the BRCA2 C-terminal region bound to DSS1 revealed five distinct BRCA2 domains (Yang *et al*, 2002). The first consists mostly of α-helices, followed by three oligonucleotide/oligosaccharide-binding (OB) folds, also present in single-stranded DNA (ssDNA)-binding proteins, such as replication protein A. The fifth domain is a tower-like structure extending from OB2, which has been inferred to bind double-stranded DNA (dsDNA). These domains possibly allow BRCA2 to recognize and bind to the ssDNA and dsDNA structure created at a DNA break site. DSS1 has been suggested to facilitate this process by mimicking ssDNA when bound to BRCA2 (Yang *et al*, 2002). Further insight into the function of DSS1 came from studies in the fungus *Ustilago maydis*, where inactivation of DSS1 was observed to give a phenotype virtually identical to mutation of the *U. maydis* BRCA2-related gene *brh2* (Kojic *et al*, 2002, 2003). This work implicated DSS1 in the repair of DSBs by homologous recombination. Here we show that in mammalian cells, deficiency of DSS1 gives a similar phenotype to that of deficiency of BRCA2, potentially implicating DSS1 in DSB break repair and cancer susceptibility.

RESULTS

DSS1 silencing results in a defect in clonogenic capacity

RNA interference (RNAi) was used to investigate the function of DSS1 in mammalian cells. We constructed plasmids (pSUPER–eCFP–hDSS1 and pSUPER–eCFP–mDss1) designed to express short interfering RNAs (siRNAs; Brummelkamp *et al*, 2002) targeting either human or mouse *DSS1* mRNAs. These were co-transfected with plasmids expressing enhanced green fluorescent protein (eGFP) fused with human or mouse DSS1 into human MCF7 cells or mouse embryonic stem (ES) cells, respectively. Silencing of mouse or human *DSS1* caused a significant reduction in the level of transfected DSS1 protein compared with cells transfected with a pSUPER plasmid expressing a control siRNA (pSUPER–eCFP–NS) as measured by western blotting (Fig 1A). Previous studies (Marston *et al*, 1999; Wei *et al*, 2003) have

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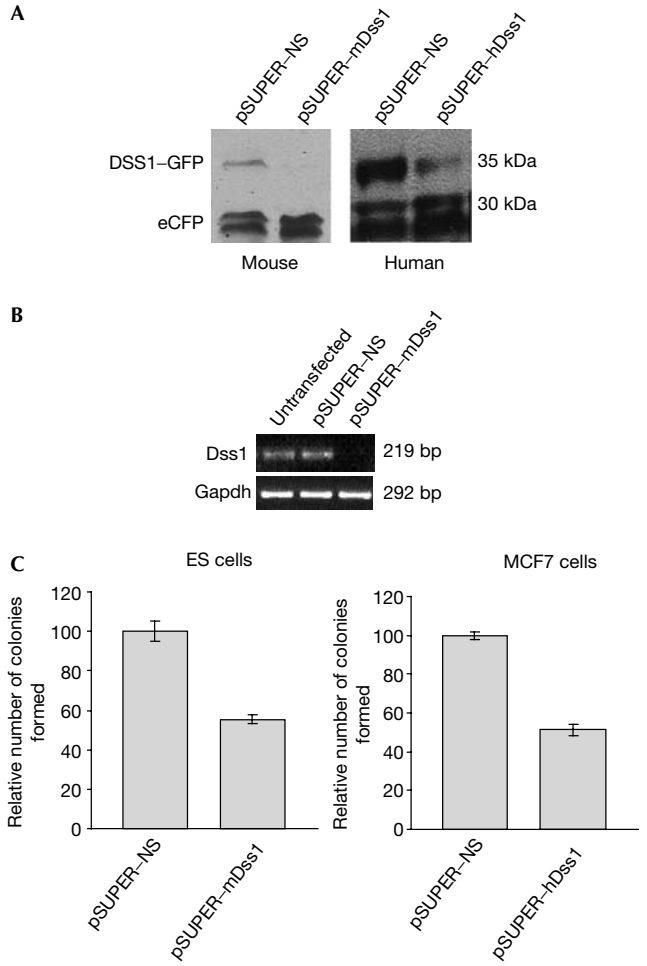

Fig 1 | Silencing of DSS1 using RNAi leads to a defect in clonogenic capacity in mammalian cells. (**A**) Reduction of DSS1 expression by RNAi. Mouse embryonic stem (ES) cells and MCF7 cells were co-transfected with vectors expressing mouse or human DSS1–eGFP fusion proteins and vectors expressing eCFP and siRNAs specific for mouse or human *DSS1* (pSUPER–eCFP–mDss1 and pSUPER–eCFP–hDSS1). Cells were also transfected with a control vector (pSUPER–eCFP–NS). At 24 h after transfection, cell lysates were prepared and analysed by western blotting. Blots were probed with anti-GFP antiserum, which recognizes both GFP and CFP. The CFP expressed from the pSUPER vector acts as a transfection and loading control. (**B**) Reduction of endogenous *Dss1* mRNA levels measured by RT–PCR. ES cells transfected with pSUPER–mDss1 were analysed after 24 h and compared with *Dss1* mRNA levels in ES cells transfected with pSUPER–NS and in untransfected cells. Levels of *Gapdh* were used as a control. (**C**) Colony formation was analysed in ES cells and MCF7 cells transfected with either pSUPER–NS or pSUPER–mDss1/pSUPER–hDSS1, respectively, together with a blasticidin resistance-encoding plasmid (pEF-Bsd). Transfection efficiencies were approximately 70%. Cells were maintained under antibiotic selection for 10–14 days and then stained with crystal violet and colonies counted. The graph shows the average percentage of colonies formed by cells transfected with pSUPER–mDss1/pSUPER–hDSS1 compared with pSUPER-NS-transfected cells (corrected to 100%). The error bars represent standard error of the mean.

documented difficulties in detecting DSS1 protein on western blots using antibodies. Therefore, to determine whether expression of pSUPER–mDss1 resulted in reduction of *Dss1* mRNA, we performed semiquantitative reverse transcriptase–PCR (RT–PCR). This demonstrated a significant reduction in *Dss1* mRNA in embryonic stem cells after transient expression of pSUPER–mDss1 compared with pSUPER–NS (Fig 1B).

We determined the effects of silencing *DSS1* expression on the ability of cells to form colonies using a clonogenic colony formation assay. ES cells and MCF7 cells were co-transfected with either pSUPER–mDss1 or pSUPER–hDSS1 along with a plasmid carrying a selectable blasticidin resistance marker. The number of colonies formed was compared with cells transfected with pSUPER–NS. This demonstrated that the clonogenic capacity of cells with reduced levels of DSS1 was significantly compromised (Fig 1C).

DSS1 silencing confers chromosomal instability

Cells deficient in the repair of DSBs by homologous recombination, such as BRCA2-deficient cells, show a high degree of chromosomal instability, including DNA breaks and radial chromosomes (Tutt & Ashworth, 2002; Venkitaraman, 2002). Given that DSS1 binds to BRCA2, we examined whether cells with silenced *DSS1* show a similar phenotype. ES cells transfected with pSUPER–mDss1 or pSUPER–NS were treated with a low dose of mitomycin C and metaphase spreads were prepared. Silencing of *Dss1* resulted in increased formation of chromosome aberrations, including chromatid/chromosome breaks (Fig 2A) and complex chromosome rearrangements (Fig 2B), including triradial and quadriradial chromosomes. Of the 50 metaphase spreads examined from pSUPER–mDss1-transfected ES cells, 23 (46%) showed chromosome aberrations compared with 13 (26%) from cells expressing pSUPER–NS. Silencing of *Dss1* resulted in a significantly higher number of chromatid/chromosome breaks per metaphase compared with cells transfected with pSUPER–NS ($P = 0.013$), with an average of 0.82 breaks compared with 0.28, respectively (Table 1). A similar trend, although not quite statistically significant, was observed for complex chromosome rearrangements ($P = 0.056$), with an average of 0.24 rearrangements (pSUPER–mDss1) compared with 0.08 (pSUPER–NS). These results suggest that Dss1 function is required for the maintenance of genomic stability in the presence of mitomycin C and loss of function leads to a similar phenotype as loss of BRCA2.

DSS1 is required for RAD51 focus formation

Following DNA damage, BRCA2 localizes with RAD51 in nuclear foci, which presumably represent sites of DSB repair by homologous recombination. Formation of these foci is dependent on wild-type BRCA2; therefore, we investigated whether this process also required DSS1. ES and MCF7 cells were transfected with pSUPER–mDss1 and pSUPER–hDSS1, respectively, then irradiated and immunostained with an antibody detecting RAD51. Cells exhibiting more than five RAD51 foci per cell were compared with cells transfected with pSUPER–NS (Fig 3A,B). We found that suppressing DSS1 in either mouse or human cells significantly reduced the number of RAD51 foci formed, suggesting that DSS1 is important for the repair of DNA by homologous recombination. To exclude the possibility that lack of DSS1 prevents DNA damage formation, we documented the formation

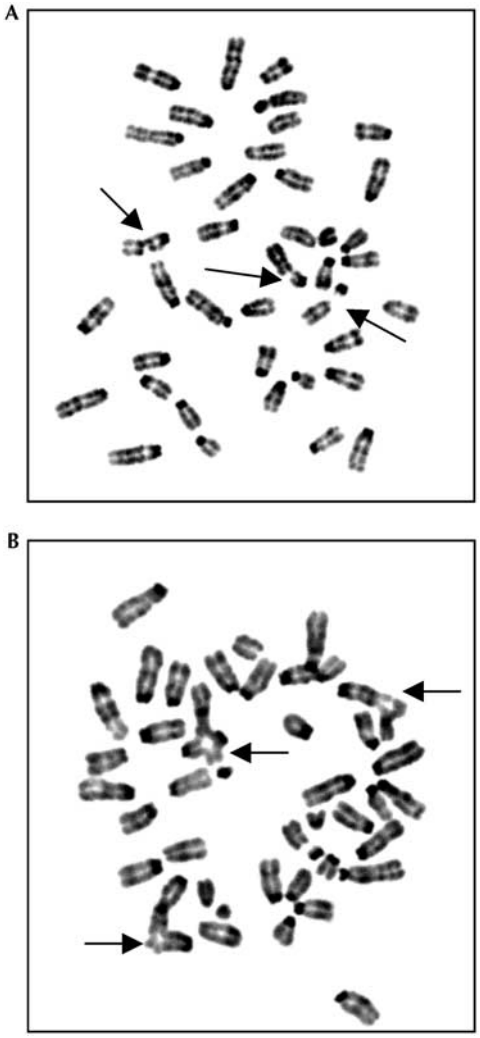

Fig 2 | DSS1 silencing confers chromosomal sensitivity to mitomycin C in mammalian cells. Embryonic stem cells, transfected with pSUPER–NS or pSUPER–mDss1, were treated with mitomycin C (0.2 μg/ml) 48 h after transfection and chromosomes were harvested the next day. Arrows in panels (**A**) and (**B**) indicate chromatid/chromosome breaks and complex chromosome rearrangements, respectively.

of γH2AX foci, which form at sites of DNA damage, DSBs and arrested replication forks (Furuta *et al*, 2003). These foci were formed equally well in DSS1-depleted or control cells (supplementary Fig 1 online). To verify that the lack of RAD51 foci formation in DSS1-deficient cells was not due to cells not entering S phase, cells were labelled with 5-bromodeoxyuridine 24 h after transfection and their cell cycle profile was analysed by fluorescence activated cell sorting (FACS; supplementary Fig 2 online). No difference was observed in the cell cycle profile of pSUPER–mDss1-transfected ES cells compared with cells transfected with pSUPER–NS. RAD51 foci have been reported to form in S phase, in some cells, independent of functional BRCA2 and in the absence of DNA damage (Tarsounas *et al*, 2003). We do not know whether DSS1 is required for the formation of these particular RAD51 foci, as they do not seem to form in the ES cells utilized for these studies (data not shown).

Table 1 | DSS1 silencing confers chromosomal sensitivity to mitomycin C

	pSUPER–mDss1	pSUPER–NS
Mean number of chromatid/chromosome breaks per metaphase ($n = 50$) ± s.e.m.	0.82 ± 0.20	0.28 ± 0.08
P-value	0.013	
Mean number of complex chromosome rearrangements per metaphase ($n = 50$) ± s.e.m.	0.24 ± 0.07	0.08 ± 0.04
P-value	0.056	

BRCA2 and RAD51 interact in DSS1-depleted cells

As demonstrated above, DSS1 seems to be required for the formation of RAD51 foci after DNA damage, which is a BRCA2-dependent process. Therefore, we investigated whether depletion of DSS1 affected BRCA2 or RAD51 protein levels or the ability of BRCA2 to associate with RAD51. We previously described (Tutt *et al*, 2001) ES cells that have a myc-tagged endogenous *Brca2* allele. These cells were transfected with pSUPER–eGFP–mDss1 or pSUPER–eGFP–NS and protein extracts prepared. The transfection efficiency of these cells was approximately 70% as measured by GFP fluorescence in a FACS analysis (data not shown). Western blot analysis demonstrated that Brca2 and Rad51 protein levels were unaffected by Dss1 depletion (Fig 4), irrespective of irradiation, suggesting that Dss1 does not influence the stability of Brca2 or Rad51. To determine whether Dss1 affects the binding of Brca2 to Rad51, Brca2 was immunoprecipitated and associated proteins were analysed by western blotting with an anti-Rad51 antibody. This experiment indicated that a similar amount of Rad51 was bound to Brca2 even when Dss1 was depleted, suggesting that Dss1 is not required for the interaction of Rad51 and Brca2.

DISCUSSION

Our results allow a refinement of a model previously described for the role of DSS1 in homologous recombination (Kojic & Holloman, 2004). This model suggested a role for DSS1 either in RAD51 binding to BRCA2 or in activating the BRCA2–RAD51 complex in response to DNA damage. We have shown that although DSS1 is unlikely to be required for the BRCA2–RAD51 interaction, it is essential for RAD51 focus formation. This could be because BRCA2 is not able to recognize or bind to DNA DSBs or because BRCA2 is unable to facilitate the assembly of RAD51 onto ssDNA. DSS1 bound to BRCA2 has been speculated to mimic ssDNA due to its acidic nature. This might facilitate accessibility of DNA-binding residues in the BRCA2 protein, allowing it to bind to DNA (Yang *et al*, 2002). The absence of DSS1 could therefore compromise the BRCA2–DNA interaction required for RAD51 oligomerization at the break site. However, a suggested role for DSS1 in stabilizing BRCA2 expressed in insect cells does not seem to be the case in mammalian cells (Yang *et al*, 2002). In

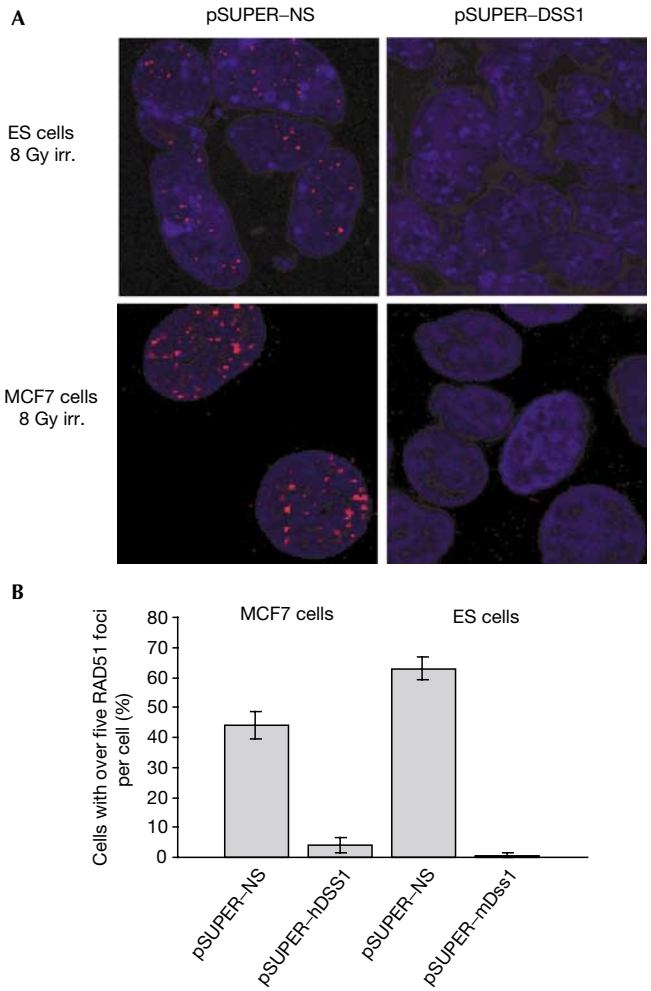

Fig 3 | DSS1 is required for RAD51 focus formation after DNA damage. (**A**) Embryonic stem (ES) cells and MCF7 cells were transfected with pSUPER–NS or pSUPER–mDss1/pSUPER–hDSS1, respectively. After 24 h, the cells were irradiated and 5 h later were fixed and stained for RAD51 foci as described by Tarsounas *et al* (2003). Nuclei are shown in blue and RAD51 foci in red. (**B**) The percentage of cells with >5 foci was quantified. The error bars represent s.e.m.

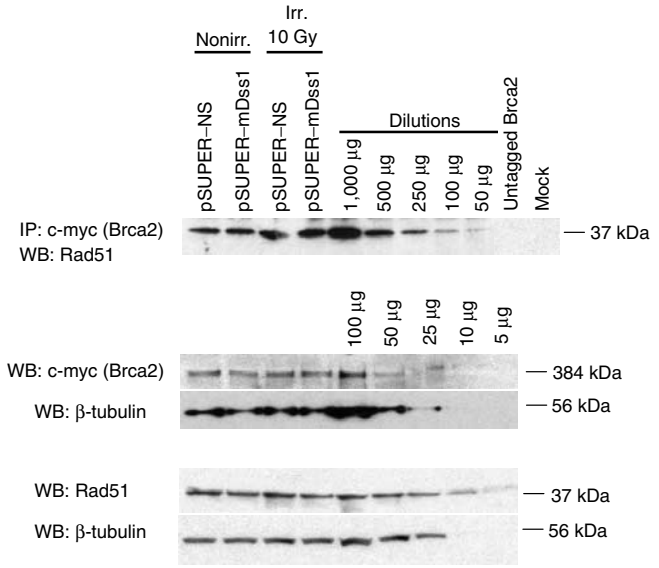

Fig 4 | DSS1 is not required for BRCA2 and RAD51 stability or the interaction of BRCA2 and RAD51. Embryonic stem (ES) cells were transfected with either pSUPER–eGFP–NS or pSUPER–eGFP–mDss1 and expression levels of myc-tagged Brca2 and Rad51 were analysed after 24 h by western blotting. Blots were probed with either anti-c-myc (A-14) antibody for Brca2 levels or anti-Rad51 Ab-1 (3C10) antibody. Blots were stripped and re-probed with anti-β-tubulin antibody as a loading control. To analyse the interaction between Brca2 and Rad51, lysates were incubated with protein G–Sepharose beads and with c-myc antibody (Brca2) or anti-GFP antiserum (mock) and bound proteins were analysed by western blotting. Lysate from ES cells with untagged Brca2 was used as a control for the immunoprecipitation. Blots were probed with anti-Rad51 antibody. Each blot also shows a titration of lysate from untransfected ES cells to demonstrate the detection level.

conclusion, we have demonstrated that loss of DSS1 creates a BRCA2-like phenotype in mammalian cells. This raises the possibility that DSS1 dysfunction, through mutation or loss of expression, could be implicated in cancer susceptibility or pathogenesis.

METHODS

Cell culture and transfection. MCF7 cells were maintained in RPMI 1640 medium supplemented with L-glutamine (GibcoBRL, Paisley, UK), 10% fetal calf serum (PAA Laboratories Ltd, Somerset, UK), penicillin/streptomycin and 10 µg/ml insulin (both from Sigma-Aldrich, Gillingham, UK). ES cells were maintained on gelatin-coated plates in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (GibcoBRL), L-glutamine, penicillin/streptomycin, β-mercaptoethanol (all from Sigma), nonessential amino acids (GibcoBRL) and ESGRO LIF (Chemicon, California, USA). The ES cells (*Brca2*$^{Tr/Ex27+}$) have been described previously (Tutt *et al*, 2001). Transfection was performed in six-well plates using FuGENE (Roche, Welwyn, UK) for MCF7 cells and Lipofectamine 2000 (Invitrogen, Paisley, UK) for ES cells according to the manufacturer's instructions.

Plasmids. DSS1–eGFP constructs were generated by subcloning PCR-amplified full-length mouse and human *DSS1* coding sequences into pEGFP-N1 (Clontech, BD Biosciences, California, USA). pSUPER (Brummelkamp *et al*, 2002) constructs were generated by expressing the following gene-specific RNAi target sequences: (i) mouse *Dss1* 5′-TGTAGAAGATGACTTCTCT-3′, (ii) human *DSS1* 5′-TGTAGAGGATGACTTCTCT-3′ and (iii) nonspecific (NS) control 5′-CATGCCTGATCCGCTAGTC-3′. These constructs also expressed a fluorescent marker, either eCFP (subcloned from pECFP–Mito (Clontech, BD Biosciences)) or eGFP (subcloned from PCAGGS EGFP–Cre described by Tutt *et al*, 2001), which had been cloned into the *Sap*I site of the pSUPER construct. These markers were used to estimate transfection efficiency using a fluorescent microscope.

Western blots. Total cell lysates were made 24 h after transfection by lysis of cells in 20 mM Tris (pH 8), 200 mM NaCl, 1 mM EDTA,

0.5% (v/v) NP-40, 10% (v/v) glycerol and protease inhibitors. For analysis of GFP fusion proteins, lysates were electrophoresed on Novex 10% Tris–glycine pre-cast gels (Invitrogen), immunoblotted with anti-GFP antiserum (Invitrogen), followed by incubation with anti-rabbit IgG-HRP and chemiluminescent detection (ECL, Amersham, UK). For myc–Brca2 western blots, 50 µg of lysate was electrophoresed on NuPAGE 3–8% Tris-acetate pre-cast gels (Invitrogen) and blots were probed with anti-c-myc (A-14) rabbit polyclonal antibody (Santa Cruz, California, USA), followed by incubation with anti-rabbit IgG-HRP and chemiluminescent detection (ECL, Amersham, UK). Rad51 was detected with Ab-1 (3C10) mouse monoclonal antibody (Neomarkers, California, USA). For co-immunoprecipitation of myc–Brca2 and Rad51, 500 µg of lysate was incubated with protein G–Sepharose beads (Sigma-Aldrich) and c-myc (A-14) goat polyclonal antibody (Santa Cruz, California, USA) or anti-GFP antiserum (Invitrogen) as a control. The same lysate was used for all the blots.

RT–PCR. RNA from ES and MCF7 cells was extracted 24 h after transfection using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. A 5 µg portion of total RNA was used for cDNA synthesis using SuperScript™ III First Strand Synthesis System for RT–PCR (Invitrogen) in a volume of 20 µl. A 0.05 µl portion of this was used as a template in PCR containing 0.2 mM of each primer, 1.5 mM $MgCl_2$, 0.25 mM of each dNTP and 1.25 U of AmpliTaq DNA polymerase (Applied Biosystems, California, USA). The following primers were used to amplify part of the *Dss1* coding region: (i) mouse Dss1F 5′-GCCTCGAGATGTCTGAAAA GAAGCAGC-3′ and (ii) mouse Dss1R 5′-CGACCGGTGATG TCTCCATCTTGTAG-3′. As a control, *Gapdh* was amplified in parallel using primers described by Shillingford *et al* (2002). The amplification conditions were 94 °C for 2 min, followed by 25 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s and a final step at 72 °C for 5 min.

Colony formation assays. ES and MCF7 cells were co-transfected with a pSUPER construct and a vector expressing blasticidin (pEF-Bsd, Invitrogen). After 24 h, the cells were trypsinized and re-seeded in six-well plates, with blasticidin treatment starting the next day and the cells re-fed every 3 days. After 10–14 days, the cells were fixed in methanol, stained with crystal violet and the colonies counted.

Fluorescence activated cell sorting analysis. To measure transfection efficiency, pSUPER–eGFP–RNAi-transfected ES cells were trypsinized and resuspended in phosphate-buffered saline with TOPRO-3 iodide (Molecular Probes, Paisley, UK) to stain dead cells and the number of GFP-positive cells was counted. FACS analysis was carried out on a FACSCalibur (Becton Dickinson, New Jersey, USA) using CellQuest software.

RAD51 focus detection. Cells were irradiated with 8 Gy the day after transfection, and 5 h later were fixed in 4% (w/v) paraformaldehyde, permeabilized with 0.2% (v/v) Triton X-100 in phosphate-buffered saline and stained with a 1:100 dilution of rabbit anti-Rad51 polyclonal antibody (BD Pharmingen, USA). After washing, the primary antibody was visualized with Alexa 555 goat anti-rabbit IgG and nuclei were visualized with TOPRO-3 iodide (Molecular Probes). Foci were observed using a Leica TCS-SP2 confocal microscope.

Mitotic chromosome analysis. At 48 h after transfection, ES cells were treated with 0.2 µg/ml mitomycin C (Sigma-Aldrich) for 1 h, followed by colcemid (0.05 µg/ml; KaryoMAX, GibcoBRL) 72 h post-transfection and metaphase spreads were prepared. Chromosomes were stained with 4′,6-diamidino-2-phenylindole and images were captured using Smart capture 2 software (Digital Scientific Ltd, Cambridge, UK) on an Axioplan 2 microscope (Zeiss, Welwyn, UK). Chromosome aberrations from each metaphase spread were counted by two individuals.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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