Vol 462|17 December 2009|doi:10.1038/nature08593

ARTICLES

The SUMO modification pathway is involved in the BRCA1 response to genotoxic stress

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Mutations in *BRCA1* are associated with a high risk of breast and ovarian cancer. BRCA1 participates in the DNA damage response and acts as a ubiquitin ligase. However, its regulation remains poorly understood. Here we report that BRCA1 is modified by small ubiquitin-like modifier (SUMO) in response to genotoxic stress, and co-localizes at sites of DNA damage with SUMO1, SUMO2/3 and the SUMO-conjugating enzyme Ubc9. PIAS SUMO E3 ligases co-localize with and modulate SUMO modification of BRCA1, and are required for BRCA1 ubiquitin ligase activity in cells. *In vitro* SUMO modification of the BRCA1/BARD1 heterodimer greatly increases its ligase activity, identifying it as a SUMO-regulated ubiquitin ligase (SRUbL). Further, PIAS SUMO ligases are required for complete accumulation of double-stranded DNA (dsDNA) damage-repair proteins subsequent to RNF8 accrual, and for proficient double-strand break repair. These data demonstrate that the SUMOylation pathway plays a significant role in mammalian DNA damage response.

The amino (N) terminus of BRCA1 has a RING domain that interacts with ubiquitin-conjugating enzymes and is required for its ubiquitin ligase activity[1–3]. Many disease-causing mutations are found within this region, and loss of the ligase activity is associated with susceptibility to breast cancer[4]. We have previously shown that BRCA1-dependent ubiquitin conjugates are generated at sites of DNA damage repair in human cells[5]. Although the substrate(s) of the BRCA1 ubiquitin ligase activity remains controversial[6], and its role at this location unclear[7], the ligase activity itself is highly conserved and damage-associated ubiquitin conjugates are also formed by *Caenorhabditis elegans*[8] and *Gallus gallus*[9] homologues of BRCA1.

BRCA1 recruitment to chromatin at sites of DNA damage occurs through a complex cascade of protein modifications and interactions, and BRCA1 is the third in a sequence of ubiquitin ligases recruited to such sites[10]. Binding of the mediator of DNA damage checkpoint 1 (MDC1) protein to the phosphorylated tail of histone H2AX (γ-H2AX) at sites of DNA breakage recruits the ubiquitin ligase RNF8[11–13], which generates ubiquitin chains bound by RAP80:ABRA1, which in turn recruits BRCA1 through its carboxy (C) terminus[14–20]. The activity of the second ubiquitin ligase, RNF168, maintains the ubiquitin chain signal initiated by RNF8 and thus helps retain BRCA1 at these sites[10,21].

SUMOylation of substrates is catalysed by a cascade of enzymes: the activities of the E1 SUMO-activating enzyme (SAE1/SAE2), the E2-conjugating enzyme (Ubc9) and E3 SUMO ligases result in an isopeptide bond between the target lysine and the activated SUMO carboxyl terminus (reviewed in ref. 22). In vertebrates, three SUMO isoforms, SUMO1, SUMO2 and SUMO3, are expressed. SUMO2 and SUMO3 differ by three N-terminal residues, and form a distinct subfamily known as SUMO2/3. Ubc9 and SUMO E3 enzymes have been implicated in the DNA damage response in human cells and animal models[23–27]. In *C. elegans*, ce-ubc9 interacts with ce-Bard1, the N-terminal binding partner of ce-Brca1[28]. The interaction of BRCA1 with free, non-conjugated SUMO1 has been shown to decrease its transcriptional activity[29]. Here we investigated the previously unaddressed role of the SUMO pathway in the regulation of BRCA1 and in the DNA damage response.

SUMO conjugation in response to damage

We noted that after treatments with genotoxic agents (irradiation, cisplatin and hydroxyurea), SUMO isoforms, SUMO1 and SUMO2/3 localized to sub-nuclear damage repair foci marked with γ-H2AX and BRCA1 (Fig. 1a–c, Supplementary Fig. 2a and data not shown). We also saw increased fluorescence resonance energy transfer (FRET) between green fluorescent protein (GFP)–BRCA1 and red fluorescent protein (RFP)–SUMO1, measured by fluorescence lifetime imaging microscopy (FLIM), after treatment of cells with the genotoxic agents cisplatin, irradiation, hydroxyurea and epirubicin (Supplementary Fig. 2b, c), indicating increased protein–protein interaction[30–34]. Increased FRET populations were observed in regions largely coincident with γ-H2AX foci in hydroxyurea-treated cells, indicating BRCA1–SUMO1 interaction at or close to γ-H2AX-decorated chromatin (Fig. 1d). The interaction was dependent on conjugation, as a SUMO mutant with a C-terminal di-glycine substitution (GG to GA) exhibited lower FRET with GFP–BRCA1 (Fig. 1e, f). Consistent with this, immunoprecipitation of endogenous BRCA1 from hydroxyurea-treated cells co-purified high molecular mass endogenous SUMO conjugates (Supplementary Fig. 2d), suggesting either SUMOylation of BRCA1 itself or interaction of BRCA1 with large, SUMO-modified proteins. *De novo* SUMOylation at sites of DNA damage would require localization of the SUMO-conjugating enzyme, Ubc9, to these sites. We found that Ubc9–GFP co-localized both with

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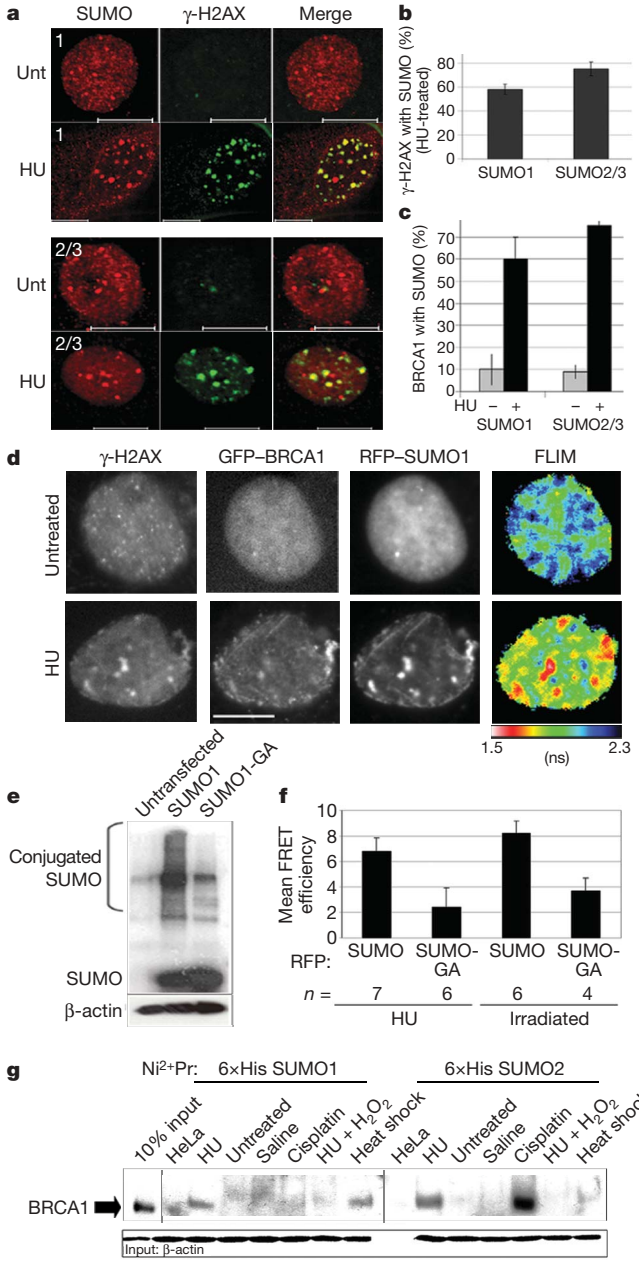

Figure 1 | The SUMO-conjugation machinery locates to sites of DNA damage, and BRCA1 is modified by SUMO after genotoxic stress. COS-7 cells with (HU) or without (Unt) hydroxyurea treatment stained with anti-SUMO isoforms and γ-H2AX (**a**) or BRCA1 and counted in **b** and **c**. Error bars, s.d.; $n > 30$ cells per condition. Scale bars, 10 μm throughout the figures. **d**, BRCA1–SUMO1 interaction at sites of genotoxic stress. Images of γ-H2AX, GFP and RFP multiphoton intensity and FLIM in transfected cells with or without hydroxyurea. FRET shortens the GFP-fluorescence lifetime in orange to red. **e**, Myc-SUMO1-GA conjugates poorly compared with wild-type myc-SUMO1 in denaturing SDS–polyacrylamide gel electrophoresis immunoblotted with anti-myc. **f**, FRET efficiency of RFP-SUMO1-GA or RFP-SUMO1 with GFP–BRCA1 in treated cells. Bars, s.e.m. **g**, SUMOylation of BRCA1. Nickel precipitation (Pr, Ni^{2+}) from untransfected (HeLa) and 6×His-SUMO-expressing cells treated with the agents shown.

γ-H2AX and BRCA1 in treated cells and showed increased FRET with RFP–BRCA1 (Supplementary Fig. 2e, f).

These data are consistent with modification of BRCA1 and/or closely associated proteins. As SUMO proteins are covalently linked to target proteins, we purified hexahistidine (6×His)-tagged SUMO conjugates using nickel-charged beads in highly denaturing conditions, and probed for BRCA1. BRCA1 was enriched on nickel after cell treatment with hydroxyurea, cisplatin and heat shock but was absent from untreated or untransfected cells, or cells treated with hydroxyurea and then low concentration H_2O_2 (Fig. 1g). (H_2O_2 cross-links Ubc9 and SAE2, preventing SUMO conjugation in HeLa cells[35], indicating that like most known SUMO targets, the modification of BRCA1 is rapidly processed.) Both SUMO isoforms conjugate to BRCA1, but more BRCA1 was purified with SUMO2 than SUMO1, particularly after cisplatin treatment. The effect of genotoxic agents on total cellular SUMO conjugates is shown in Supplementary Fig. 2g.

Together, these data indicate that the SUMO conjugation pathway forms part of the mammalian response to DNA damage, as Ubc9 and the SUMO proteins interact with at least one DNA damage-regulated SUMOylation target, BRCA1, at sites of genotoxic stress labelled by γ-H2AX.

PIAS SUMO ligases in the damage response

Members of the protein inhibitor of activated signal transducer and activator of transcription (PIAS) family of SUMO E3 ligases and the Mms21(NSE2) SUMO ligase are found in foci within the nucleus[36], and have been reported to play a role in the DNA damage response[24–27]. Short interfering RNA (siRNA) depletion of PIAS1 and PIAS4 impaired 6×His SUMO1 and SUMO2 modification of endogenous BRCA1 in hydroxyurea-treated cells, unlike depletion of MMS21, PIAS2 or PIAS3 (Fig. 2a and data not shown). In untreated cells, depletion of PIAS1 resulted in increased SUMO2-conjugated BRCA1: this was dependent on PIAS4 because depletion of PIAS1 and PIAS4 inhibited the modification (Fig. 2a). Quantitative PCR with reverse transcription (RT–PCR) showed that siRNAs to each SUMO ligase were E3 specific, and ligase depletion had no impact on steady-state BRCA1 protein levels (Supplementary Fig. 3a, b). Ectopic expression of PIAS1 and PIAS4 (but not MMS21) increased BRCA1–SUMO interaction, as measured by FLIM (Fig. 2b) and 6×His-SUMO-conjugated BRCA1 (Fig. 2c). Increased expression or depletion of PIAS ligases had no obvious impact on total SUMO1 conjugates, but SUMO2 conjugates (in 6×His-SUMO2 cells) were decreased on PIAS1 depletion, and increased after ectopic expression of PIAS1 and PIAS4 (Fig. 2a and

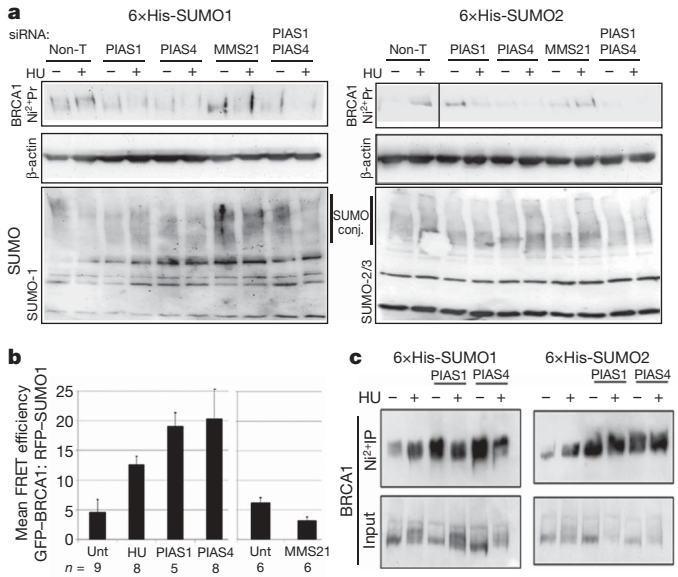

Figure 2 | PIAS E3 SUMO ligases modulate BRCA1 SUMOylation. **a**, PIAS ligases are required for BRCA1 SUMOylation. Nickel elutant from untreated or hydroxyurea-treated 6×His-SUMO-expressing cells transfected with siRNA to transcripts shown (Non-T, non-targeting) and probed with antibodies shown. SUMO conj, SUMO conjugates. **b**, PIAS ligases increase BRCA1–SUMO1 interaction. FRET efficiency of RFP-SUMO1 with GFP–BRCA1 in untreated (Unt) cells or transfected with SUMO ligases shown, or cells treated with hydroxyurea. Bars, s.e.m. **c**, PIAS ligases increase BRCA1 SUMOylation. Nickel elutant from 6×His-SUMO-expressing cells transfected with PIAS expression constructs.

Supplementary Fig. 3c). Thus BRCA1 is one of many substrates modified by SUMO2 in response to PIAS activity but is one of a smaller population of SUMO1 substrates regulated by PIAS proteins.

We assessed the impact of SUMO-ligase depletion on BRCA1 localization in cells and found that loss of PIAS1 and PIAS4, but not MMS21, PIAS2 or PIAS3 SUMO ligases, reduced its ability to localize to γ-H2AX in hydroxyurea-treated cells (Fig. 3a, b and data not shown). The introduction of siRNA-resistant PIAS proteins at low expression levels restored the ability of endogenous BRCA1 to co-localize with γ-H2AX (Supplementary Fig. 4a). PIAS1, PIAS4 and MMS21 ligases expressed in cells co-localized with BRCA1. High expression of PIAS1 or PIAS4, but not MMS21, together with BRCA1 and SUMO1 or SUMO2, resulted in exaggerated GFP–BRCA1 foci (Supplementary Fig. 4c). The formation of exaggerated foci was dependent on the SUMO ligase activity, because an inactive PIAS1 RING mutant[37] inhibited the ability of PIAS1 both to induce increased BRCA1–SUMO1 interaction in cells (Supplementary Fig. 4b) and to cause exaggerated BRCA1 foci (Supplementary Fig. 4c, d). Together, these data indicate that PIAS1 and PIAS4 SUMO ligases modulate and are required for normal accumulation of BRCA1 at sites of genotoxic stress.

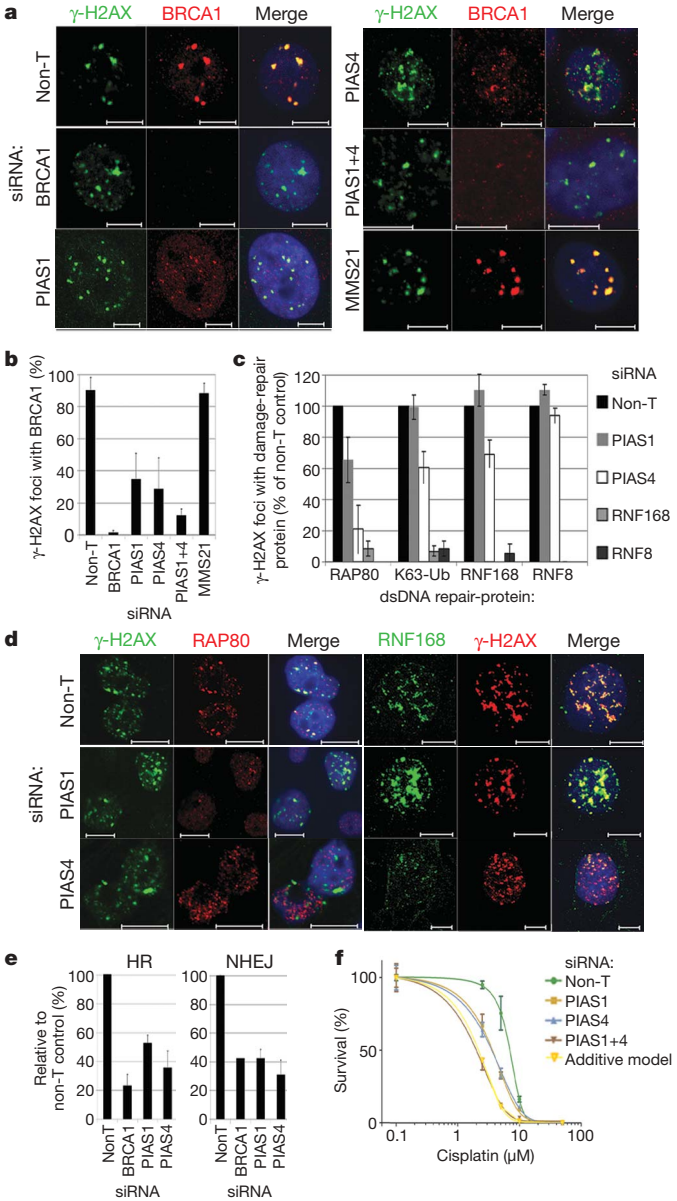

Figure 3 | PIAS E3 SUMO ligases influence accumulation of DNA damage-repair protein and are required for dsDNA break repair. a, BRCA1 accumulation to γ-H2AX in hydroxyurea-treated cells transfected with siRNAs indicated and counted in **b** (bars, s.d.; $n > 30$ cells per condition). **c**, Hydroxyurea and siRNA treated cells scored for γ-H2AX foci with accumulated dsDNA damage proteins (bars, s.d.; $n > 30$ cells per condition) and representative immunofluorescence images shown in **d**. **e**, Homologous recombination (HR) and non-homologous end joining (NHEJ) assayed in siRNA transfected cells bearing integrated gene conversion and end-joining substrates after I-sceI-induced dsDNA break. Bars, s.d. **f**, Colony survival of siRNA-transfected cells exposed to cisplatin. Combined sensitivity of PIAS1 + PIAS4 was characterized as additive by the Bliss independence model[50].

SUMO conjugation influences BRCA1 ligase

To examine whether impaired BRCA1 accumulation following PIAS depletion is likely to be direct or indirect, we investigated the integrity of the upstream accumulation cascade by examining RAP80, K63-linked ubiquitin (K63-Ub), RNF168 and RNF8, all proteins necessary for BRCA1 recruitment to sites of genotoxic stress[10–14,16,18–21]. PIAS4 was required for normal accumulation of proteins subsequent to RNF8, affecting RNF168, K63-Ub, RAP80 and BRCA1, whereas PIAS1 was required for complete accumulation of proteins subsequent to the generation of K63-linked ubiquitin, affecting RAP80 and BRCA1 (Fig. 3c, d and Supplementary Fig. 4e). Thus, although BRCA1 is SUMO modified in a PIAS-dependent manner, these data indicate that its accumulation is regulated through an indirect mechanism involving earlier-arriving proteins.

Consistent with the requirement on PIAS proteins for BRCA1 accumulation, depletion of these ligases, like depletion of BRCA1, reduced homologous recombination and non-homologous end-joining repair of double-strand breaks, and increased cellular sensitivity to cisplatin (Fig. 3e, f), indicating that these SUMO ligases are required for the full response to DNA damage.

In cells, ubiquitin conjugates detected by the monoclonal antibody FK2 are lost from sites of DNA damage after depletion of many proteins involved in accumulation of dsDNA repair protein, including the ubiquitin ligases RNF8, RNF168 and BRCA1 (refs 5, 8–10, 21, 38). FK2-ubiquitin accumulation to γ-H2AX was also reduced after depletion of PIAS1 and PIAS4 (data not shown). Because PIAS1 depletion does not impair RNF168/K63-Ub accumulation (Fig. 3c), this suggests that the impact on FK2-ubquitin is independent or subsequent to RNF8/RNF168 activity. FK2-ubiquitin accumulation was also reduced in the small population of PIAS-depleted cells that retained some BRCA1 foci (Fig. 4a, b), suggesting disruption of an activity subsequent to BRCA1 accumulation.

Ectopic expression of BRCA1/BARD1 in cells is able to increase FK2-ubiquitin conjugate staining above levels detected in surrounding, non-transfected cells, in a manner dependent on a functional RING domain[5]. This ability was absent in PIAS1/4-depleted cells (Supplementary Fig. 5a), indicating that the BRCA1 ubiquitin ligase activity is reduced. Similarly the co-localization of K6-linked ubiquitin (catalysed by BRCA1 (refs 5, 39, 40)) with γ-H2AX was impaired in BRCA1-, PIAS1- or PIAS4-depleted cells, consistent with loss of BRCA1 ligase activity (Supplementary Fig. 5b).

SUMO conjugation frequently, but not always, occurs on lysines in the consensus motif, 'ΨKxE', where Ψ is an aliphatic residue. The two highest-scoring motifs in BRCA1 are residues K109 and K119, adjacent to the RING domain (Fig. 4c). The interaction of BRCA1 K109→R with RFP–SUMO1 was comparable to that of wild-type BRCA1 (data not shown). However, substitutions of the K119 motif (K119→R or E121→A) reduced interaction with RFP–SUMO1 (Fig. 4d). These mutations, like the RING mutation, C61G, reduced the ability of ectopic BRCA1 to induce increased levels of co-localizing FK2 ubiquitin conjugates in cells (Fig. 4e, f), consistent with the effects of PIAS1 and PIAS4 ligase depletion.

These observations imply that the SUMOylation pathway acts directly on the BRCA1/BARD1 ligase. To test this, we explored BRCA1 SUMO modification *in vitro* (Supplementary Fig. 5c, d). Titration of unmodified against SUMO1-modified heterodimer showed that the modified form had increased ubiquitin ligase activity, generating 10–20 times more conjugated ubiquitin (Fig. 4g).

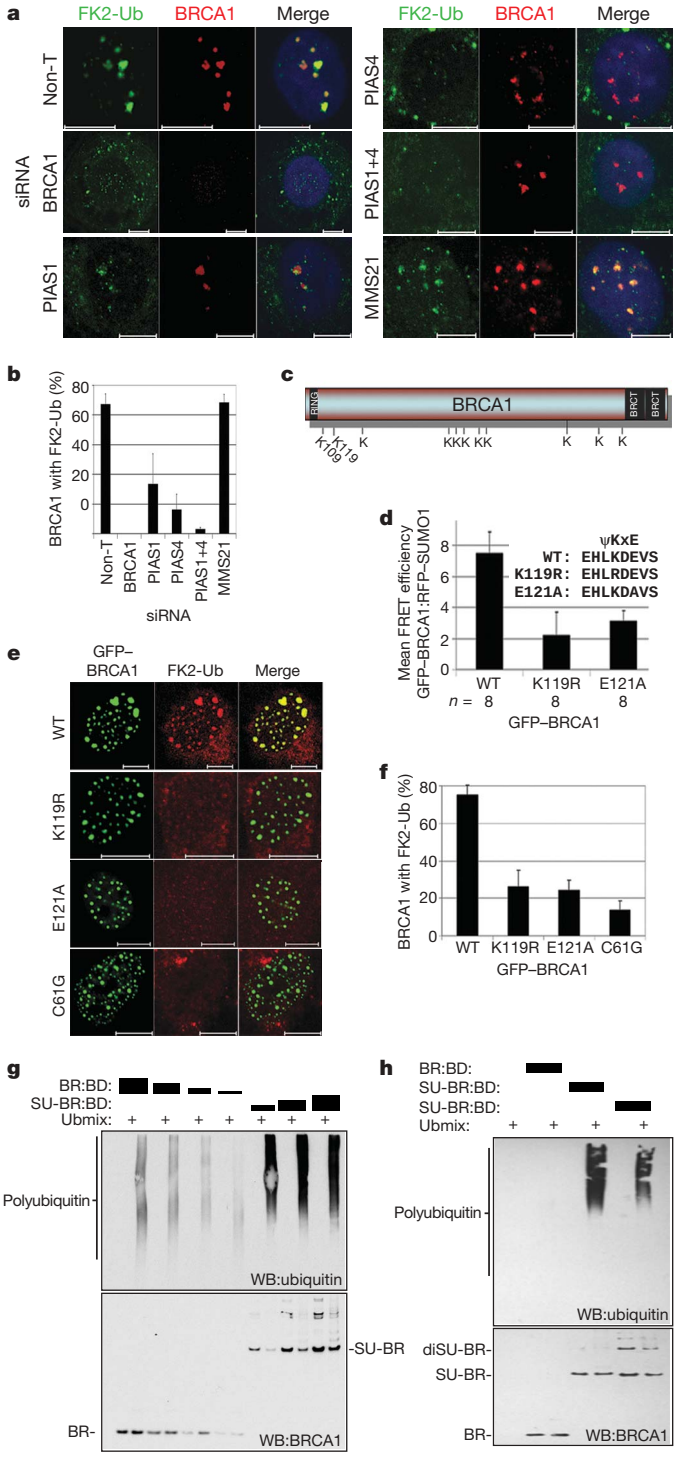

Figure 4 | The SUMO pathway regulates BRCA1 ubiquitin ligase activity. **a**, FK2-ubiquitin accumulation with BRCA1 in siRNA and hydroxyurea-treated cells and counted in **b** (bars, s.d.; $n > 30$ cells per condition). **c**, Illustration of BRCA1 motifs and consensus SUMO sites (K) identified by both abgent.com/doc/sumoplot and bioinformatics.lcd-ustc.org/sumosp programs; K119 and K109 rank highest in both. **d**, BRCA1 K119 consensus is required for interaction with SUMO1 in hydroxyurea-treated cells. Bars, s.e.m. **e**, Cells expressing SUMO consensus or RING-C61G mutants stained with low titration FK2 and scored in **f** (bars, s.d.; $n > 30$ cells per condition). WT, wild type. **g**, **h**, SUMO-modification increases the ubiquitin ligase activity of $BRCA1_{1–147}$–$BARD1_{26–142}$. BR, BRCA1; BD, BARD1; SU1, SUMO1; SU2, SUMO2. Ubmix indicates ubiquitin conjugation components except the heterodimer.

Comparison of unmodified heterodimer with SUMO1 and SUMO2 modified forms, at a concentration where the unmodified proteins had little detectable activity, showed that the SUMO modification enhanced ubiquitin ligase activity independent of isoform specificity (Fig. 4h).

Discussion

SUMO modification increases BRCA1 ubiquitin ligase activity *in vitro*, consistent with the requirement in cells for PIAS SUMO E3 ligases and for an N-terminal SUMO modification consensus site, thus identifying BRCA1 as a SUMO-regulated ubiquitin ligase (SRUbL). SUMO modification of BRCA1 and occupation of the BRCA1 RING by ubiquitin E2 conjugating enzymes are concurrent, supported by the observation that the presence of several-fold molar excess of the ubiquitin E2, UbcH5a, has little impact on BRCA1 SUMO modification *in vitro* (data not shown). Similarly, mutations that inhibit BRCA1–ubiquitin E2 interactions (T77→M, I26→A, C61→G or the absence of BARD1 polypeptide) had no impact on BRCA1 SUMOylation, indicating that the BRCA1/BARD1 heterodimer RING domains are not required for SUMO pathway interaction (data not shown). Thus the simplest mechanism envisaged is that SUMO modification of BRCA1 increases the E3–E2 interface, through SUMO interaction with the E2 enzyme (possibly through SUMO interacting motifs). Based on *in vitro* investigations, other authors[41,42] have shown that auto-ubiquitylation of BRCA1 at positions C-terminal to its RING domain regulates ligase activity and E2 choice, although how BRCA1 SUMOylation and auto-ubiquitylation relate is yet to be clarified.

These data show that the PIAS SUMO ligases are necessary components of the mammalian response to double-strand breaks, required for homologous recombination and non-homologous end-joining, and that they influence BRCA1 accumulation through earlier-arriving proteins. However, the details of their regulation by the SUMO pathway remain to be determined. It is possible, for example, that like BRCA1, the other ubiquitin ligases in the pathway, RNF8 and RNF168, are also SRUbLs regulated by SUMO-modification (Supplementary Fig. 1).

The known post-translational modifications of BRCA1 now include phosphorylation, ubiquitylation and SUMOylation. Because the two features of BRCA1 activity regulated by the SUMO pathway, ubiquitin ligase activity and accumulation at sites of DNA damage, are also inhibited by some *BRCA1* mutations that predispose to breast and ovarian cancer[4,43], it seems highly likely that the SUMO pathway will be of relevance to cancer predisposition and development.

METHODS SUMMARY

Cell treatments. Cells were treated with 10 μM cisplatin for 3 h followed by 16 h recovery, 16 h in 20 nM epirubicin, 3 mM hydroxyurea, or 3 mM hydroxyurea for 8 h followed by 1 mM H_2O_2 for 15 min, 15 min heat shock at 43 °C or exposure to 10 Gy irradiation using a caesium-137 source.

SiRNA. SMART Pool siRNAs (Dharmacon) used were: BRCA1 (L-003461-00), PIAS1 (L-008167-00), PIAS2 (L-009428-00), PIAS3 (L-004164-00) PIAS4 (L-006445-00) MMS21/NSE2 (L-018070-00), RNF168 (L007152-00-0005) and RNF8 (L-009600-00-0005), non-targeting control siRNA, confirmed to have minimal targeting of known genes (D-001810-10-05). The untranslated regions target sequences were as follows: GGCGAAGUUCACUGCGC (PIAS1), CAGAGGGAGGAGUGACC (PIAS4).

Purification of 6×His tagged SUMO conjugates. This was as described previously[44].

Immunofluorescence microscopy. This was performed as previously described[5].

Antibodies. The following antibodies were used: MS110 (Ab1, Calbiochem), FK-2 (Biomol), anti-Flag (M2) (Sigma), control rabbit IgG (Sigma), β-actin (Abcam), anti-c-myc, anti-SUMO1, anti-SUMO2/3 (Santa Cruz), γ-H2AX (Millipore and Abcam), anti-RNF8 (Abnova), anti-RNF168[10], anti-RAP80 (Bethyl) and anti-K63-ubiquitn (Millipore).

Time-resolved multiphoton microscopy. Measurements were undertaken with a modified system similar to that described previously[45]. Fluorescence lifetime imaging used time-correlated single-photon counting electronics (Becker & Hickl, SPC 830) collected through a bandpass filter centred at $\lambda = 510 \pm 10$ nm (Chroma). Excitation power was adjusted using a neutral density filter to photon counting rates ~10^4–10^5 photons s^{-1}, and acquisition times ~300 s at low excitation power were used. Imaging control and analysis used

custom software (CVI LabWindows)[46]. The Förster radius of the GFP and RFP pair used has been calculated as 4.7 nm (ref. 31).

Repair sssays. Homologous recombination and non-homologous end-joining cell assays were performed as previously descibed[47].

Protein production and ubiquitin ligase assays. These were as previously described[4].

***In vitro* SUMO conjugation assays.** These were as previously described[48,49].

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 17 May; accepted 19 October 2009.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We are grateful to V. De Laurenzi for the PIAS1 expression constructs, to R. Hay for His-SUMO1 and His-SUMO2 cells, to D. Durocher for anti-RNF168 antibody, to G. Stewart for discussions and S. Jackson for sharing results before publication. The work was supported by grants from Breast Cancer Campaign (to J.R.M., A.A., #SF06, and L.B., #06NovPHD13Morris), Cancer Research UK (to R.D., #C8820/A9494), the Medical Research Council (to E.S. and D.W., #6900577, and C.B.), the Richard Dimbleby Cancer Fund to King's College London (to M.K. and T.N.) and Breakthrough Breast Cancer (to T.K). Multiphoton FLIM systems and acquisition/analysis software were built by S. Ameer-Beg, P. Barber and B. Vojnovic, with support from MRC Co-operative Group Grant G0100152 #56891 and UK Research Councils Basic Technology Research Programme Grant GR/R87901/01.

Author Contributions J.R.M. conceived and designed the study, generated reagents, performed experiments and wrote the paper. C.B. performed *in vitro* assays, confirmed and developed the initial concept, and generated reagents. M.K. optimised and performed FLIM measurements and analysis. R.D. and D.W. performed experiments and generated reagents. L.B. performed co-localisation observations, A.A. and L.P. generated reagents and Y.G. generated reagents and participated in discussions. T.K. undertook FLIM measurements. T.N. provided expertise and input into the design of the FLIM experiments, and E.S. provided advice and mentoring to J.R.M.

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METHODS

Plasmid constructs. Full-length BRCA1, SUMO1 and SUMO1-GA were cloned 3′ of the modified RFP of pcDNA3.1 (ref. 31) or in myc-pcDNA3.1, and full-length BRCA1 and UBC9 were cloned 3′ of the GFP in p-EGFP (Clontech). Flag–HIS-MMS21 complementary DNA was cloned into pCL-NCX previously modified to contain 3×Flag–HIS tag, and PIAS4 in 3×Flag–Stag in pCDNA3.1(−) modified to contain 3Flag–Stag. PIAS1 expression constructs were gifts (see Acknowledgements). Point mutations were generated using site-directed mutagensis and confirmed by sequencing. Ubiquitin and BARD1 clones in pcDNA3.1 have been previously described[5].

Cell treatments. Cells were treated with 3 mM hydroxyurea for 8 h, 0.9% NaCl carrier, 10 μM cisplatin for 3 h followed by 16 h recovery, or 16 h in 20 nM epirubicin, 3 mM hydroxyurea for 8 h followed by 1 mM H_2O_2 for 15 min or 15 min heat shock at 43 °C or cells were exposed to 10 Gy irradiation using a Gammacell 1000 Elite irradiator (caesium-137 source).

siRNA. On-target Plus SMART Pool siRNAs (Dharmacon) used were: BRCA1 (L-003461-00), PIAS1 (L-008167-00), PIAS2 (L-009428-00), PIAS3 (L-004164-00) PIAS4 (L-006445-00) and MMS21/NSE2 (L-018070-00), RNF168 (L007152-00-0005), RNF8 (L-009600-00-0005). Non-targeting control siRNA has been confirmed to have minimal targeting of known genes (D-001810-10-05). siRNAs to untranslated regions used were to target sequence: GGCGAAG UUCACUGCGC (PIAS1), CAGAGGGAGGAGUGACC (PIAS4). Knockdowns were confirmed by RT–PCR of extracted cell-line RNA and were designed over more than one exon boundary to encompass the active site (RING).

Purification of 6×His tagged SUMO conjugates. His-SUMO stable Hela cells[44] were maintained in 0.5 μg ml^{-1} puromycin and were a gift from R. Hay. Cells were lysed directly in 8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 0.01 M Tris–HCl, pH 6.3, 10 mM β-mercaptoethanol, 5 mM imidazole plus 0.2% Triton-X-100, harvested and sonicated. They were then mixed with 50 μl of Ni^{2+} Talon agarose beads (BD-Bioscience) and incubated overnight at 4 °C, washed and eluted in SDS–polyacrylamide gel electrophoresis buffer according to ref. 44.

Immunofluorescence microscopy. All cells were grown and prepared as previously described[5].

Antibodies. The antibodies used in the study were MS110 ascites (Ab1, Calbiochem), FK-2 (Biomol), anti-BARD1 antibody (Serotec), anti-Flag (M2) (Sigma), control rabbit IgG (Sigma), β-actin (Abcam), anti-c-myc (9E10), anti-SUMO1, anti-SUMO2/3, (Santa Cruz), γ-H2AX clone JBW301 (Millipore) and polyclonal anti-γ-H2AX (Abcam), anti-RNF8 (Abnova), anti-RNF168 (ref. 10), anti-RAP80 (Bethyl) and anti-K63-ubiquitin (Millipore).

Time-resolved multiphoton microscopy. FLIM measurements were undertaken with a modified multiphoton microscopy system similar to that described previously[45]. Fluorescence lifetime imaging capability was provided by time-correlated single-photon counting electronics (Becker & Hickl, SPC 830). Data were collected through a bandpass filter centred at $\lambda = 510 \pm 10$nm (Chroma). Excitation power was adjusted using a neutral-density filter to give average photon counting rates of the order 10^4–10^5 photons s^{-1} to avoid pulse pile up. Acquisition times of the order of 300 s at low excitation power were used to achieve sufficient photon statistics for fitting, while avoiding either pulse pile up or observable photo-bleaching. The imaging system was controlled, and the data later analysed, with custom software written in CVI LabWindows[46]. FRET efficiency $= 1 - \tau da/\tau di$, where d*a* is the pixel-by-pixel fluorescence lifetime of the donor in the presence of acceptor and d*i* is the average lifetime of the donor in the absence of acceptor (in all experiments unlabelled BARD1 was co-expressed). The Förster radius (distance at which the efficiency of energy transfer is 50%) of the GFP and RFP pair has been calculated as 4.7 nm (ref. 31). Note that in analysis of FRET data there are two elements that must be considered: interacting fluorophore population and FRET efficiency. Bulk measurements of FRET efficiency cannot distinguish between an increase in FRET efficiency (that is, proximity) and an increase in FRET population (concentration of interacting species) because the two parameters are not resolved.

Repair assays. HeLa clones with the homologous recombination (DR–GFP) and total non-homologous end-joining (EJ5–GFP) reporters stably integrated were generated as previously described[47]. For repair assays, HeLa-DR–GFP or Hela-EJ5–GFP were either mock transfected or transfected with non-targeting or targeting siRNA. Cells were left for 24 h before transfection with the I-sceI expression vector pCBA-I-sceI. Three days after pCBA-I-sceI transfection, cells were fixed and the proportion of GFP-positive cells counted. Counts were performed in triplicate.

Clonogenic cell survival assays. 293T cells were plated onto 24-well tissue culture dishes at (10^5 cells per well) and transfected with siRNA and plasmid with Dharmafect according to the manufacturer's instructions. After 3 days, they were exposed to cisplatin (Sigma Chemicals) for 3 h and replated into 10-cm dishes at various concentrations. After 11 days, cells were fixed with methanol for 10 min and stained with 0.5% crystal violet (BDH Chemicals). Washed dishes were dried, and colonies >1 mm were scored. Half-maximum inhibitory concentration (IC_{50}) values were calculated for each siRNA from the respective sigmoidal dose–response curves using Prism software. The Bliss independence model is defined by the equation $Exy = Ex + Ey - (ExEy)$, where *Exy* is the additive effect of siRNA 1 and 2 as predicted by their individual effects (*Ex* and *Ey*)[50].

Protein production, SUMO conjugation and ubiquitin ligase assays. Bacterial expression of human BRCA1/BARD1 heterodimer was from bi-cistronic expression vector purified using nickel resin as described previously[4].

***In vitro* SUMO conjugation assays.** These were performed as described by Boutell *et al.*[48,49]. Purification of SUMOylated complexes is described in Supplementary Fig. 2.

Immunoprecipitation. 293T cells were lysed in 20 mM Tris-HCL, pH 8, 137 mM NaCl, 1 mM EGTA, 1% Triton-X-100, 10% glycerol, 1.5 $MgCl_2$, containing 10 mM iodoactetamide and protease inhibitors. After clearance, lysate was incubated with 20 μl MS110 (Ab1) overnight. Beads were washed in lysis buffer before elution in SDS–polyacrylamide gel electrophoresis buffer.