The interaction between CtIP and BRCA1 is not essential for resection-mediated DNA repair or tumor suppression

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T he CtIP protein facilitates homology-directed repair (HDR) of double-strand DNA breaks (DSBs) by initiating DNA resection, a process in which DSB ends are converted into 3′-ssDNA overhangs. The BRCA1 tumor suppressor, which interacts with CtIP in a phospho-dependent manner, has also been implicated in DSB repair through the HDR pathway. It was recently reported that the BRCA1–CtIP interaction is essential for HDR in chicken DT40 cells. To examine the role of this interaction in mammalian cells, we generated cells and mice that express Ctip polypeptides (Ctip-S326A) that fail to bind BRCA1. Surprisingly, isogenic lines of Ctip-S326A mutant and wild-type cells displayed comparable levels of HDR function and chromosomal stability. Although Ctip-S326A mutant cells were modestly sensitive to topoisomerase inhibitors, mice expressing Ctip-S326A polypeptides developed normally and did not exhibit a predisposition to cancer. Thus, in mammals, the phospho-dependent BRCA1–CtIP interaction is not essential for HDR-mediated DSB repair or for tumor suppression.

Introduction

At least three distinct pathways for repair of DNA double-strand breaks (DSBs) have been identified in eukaryotic cells: homology-directed repair (HDR), Ku-dependent nonhomologous end joining (NHEJ), and Ku-independent microhomology-mediated end joining (MMEJ; Symington and Gautier, 2011). In vivo, the pathway used for repair of a given DSB is governed in part by DNA resection. This nucleolytic process converts DSB ends into 3′-ssDNA overhangs that inhibit NHEJ repair, but act as essential intermediates for both HDR and MMEJ (Symington and Gautier, 2011). In addition, the 3′-ssDNA tails generated by resection are bound initially by RPA protein complexes to form ssDNA–RPA nucleoprotein filaments that trigger ATR-dependent checkpoint signaling and subsequently by Rad51 polypeptides to form the ssDNA–Rad51 filaments that mediate HDR.

As shown in yeast, DNA end resection involves at least two mechanistically distinct stages (Mimitou and Symington, 2008; Zhu et al., 2008; Nicolette et al., 2010; Niu et al., 2010; Symington and Gautier, 2011). During an initiation stage, the yeast MRX (Mre11–Rad50–Xrs1) complex, together with the Sae2 protein, mediates a limited degree of resection to yield short ssDNA tails of roughly 100–400 nucleotides. In a subsequent extension stage, ssDNA tails greater than a kilobase in length can be generated by the Exo1 exonuclease or through the coordinated action of the DNA2 endonuclease and a RecQ-family helicase. As the human orthologue of yeast Sae2, the CtIP protein collaborates with MRN (Mre11–Rad50–Nbs1) to promote DNA resection, ATR signaling, and HDR repair in mammalian cells (Sartori et al., 2007; Bennardo et al., 2008; Chen et al., 2008). Indeed, Ctip/Sae2 and their orthologues have now been implicated in DNA resection across a vast phylogenetic spectrum that encompasses fungi, plants, insects, and vertebrates (Limbo et al., 2007; Penkner et al., 2007; Uanschou et al., 2007; You et al., 2009; You and Bailis, 2010; Peterson et al., 2011).

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Supplemental Material can be found at:
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Recent studies show that CtIP/Sae2-mediated resection is also required to expose as ssDNA the microhomologies necessary for MMEJ repair of DSBs (Lee and Lee, 2007; Bennardo et al., 2008). In addition, CtIP can facilitate the conversion of chromosomal DSBs into aberrant chromosome translocations in mouse embryonic stem (ES) cells, suggesting a potential pathological role for this protein (Zhang and Jasin, 2011). In any event, as a key effector for the initiation step of DNA resection, CtIP generates essential intermediates for checkpoint signaling (ssDNA–RPA filament), HDR (ssDNA–Rad51 filament), and MMEJ (ssDNA). Apart from its well-defined role in DNA resection, CtIP has also been implicated in other cellular processes, including transcriptional regulation and cell cycle progression (Chinnadurai, 2006).

In early studies, CtIP was identified as a major in vivo partner of the BRCA1 tumor suppressor (Wong et al., 1998; Yu et al., 1998; Yu and Baer, 2000). Although germline mutations of the BRCA1 gene are a major cause of the familial breast and ovarian cancer syndrome, the mechanisms by which BRCA1 suppresses tumor formation are still unclear (Huen et al., 2010; Moynahan and Jasin, 2010; Li and Greenberg, 2012; Roy et al., 2012). BRCA1 has been implicated in multiple aspects of the DNA damage response and it plays an essential, but undefined, role in the HDR pathway of DSB repair. At its C terminus, BRCA1 harbors two tandem BRCT repeats that form a single phospho-recognition surface. Of note, the BRCT surface of BRCA1 can bind the phosphorylated isoforms of several important DNA repair proteins, including Abraxas/CCDC98, BACH1/FancJ/BRIP1, and CtIP. Because BRCA1 interacts with each of these BRCT phospho-ligands in a mutually exclusive manner, it has the potential to form at least three distinct protein complexes (BRCA1 complexes A, B, and C, respectively) that appear to influence different aspects of the DNA damage response (Yu and Chen, 2004; Greenberg et al., 2006; Kim et al., 2007; Liu et al., 2007; Wang et al., 2007).

Because the BRCA1 lesions associated with familial breast cancer are usually frameshift or nonsense mutations, most tumorogenic BRCA1 alleles encode truncated polypeptides that have lost one or both BRCT motifs (Huen et al., 2010; Moynahan and Jasin, 2010; Li and Greenberg, 2012; Roy et al., 2012). Moreover, in some breast cancer families, tumor susceptibility can be ascribed to single amino acid substitutions (e.g., the S1655F mutation) that disrupt the interaction between the BRCT domain and its cognate phospho-ligands (Botuyan et al., 2004; Clapperton et al., 2004; Shiozaki et al., 2004; Williams et al., 2004; Varma et al., 2005). Indeed, using a mouse model of hereditary breast cancer, we recently showed that BRCT phospho-recognition is essential for both the HDR and tumor suppression activities of BRCA1 (Shakya et al., 2011). Thus, these two critical functions of BRCA1 are dependent on its ability to interact with one or more of its BRCT phospho-ligands (Shakya et al., 2011).

Of the known BRCA1 phospho-ligands, CtIP is especially intriguing given its central role in DNA resection and DSB repair (Sartori et al., 2007; You and Bailis, 2010). BRCA1 specifically binds human CtIP isoforms that are phosphorylated at serine residue S327, primarily during the G2 phase of the cell cycle (Yu and Chen, 2004). At present, however, the function of the BRCA1–CtIP interaction is poorly understood. In particular, it remains unclear whether this interaction is required for the tumor suppression activity of BRCA1 and/or the DNA resection activity of CtIP. Although the former possibility has not been tested experimentally, the latter has been addressed in studies of chicken DT40 cells that express nonphosphorylatable forms of CtIP. For example, Yun and Hiom (2009) reported that CtIP-null chicken DT40 cells reconstituted with an exogenous expression vector encoding human S327A mutant CtIP (CtIP+/-/-; + hCtIP-S327A) are defective for HDR, but not NHEJ or MMEJ. On this basis, they concluded that the BRCA1–CtIP interaction is required for CtIP-mediated resection and, in turn, for DSB repair through pathways, such as HDR, that entail extensive DNA resection. In contrast, Nakamura et al. (2010) observed normal levels of HDR in DT40 cells (CtIP3323A/-/--) that express endogenous CtIP bearing the corresponding mutation (S332A) of chicken CtIP, suggesting that the BRCA1–CtIP interaction is dispensable for resection-dependent repair.

To examine this issue in mammalian cells, and to ascertain whether the BRCA1–CtIP interaction is required for BRCA1-mediated tumor suppression, we have introduced the corresponding mutation (S326A) into mouse embryonic stem (ES) cells to generate cells and mice expressing Ctip–S326A polypeptides that fail to interact with mouse Brca1. Here we show that the S326A mutation does not impair resection-dependent pathways of DSB repair (e.g., HDR and MMEJ) and does not abrogate Brca1-mediated tumor suppression. These findings indicate that, at least in mammalian cells, the BRCA1–CtIP interaction is dispensable for these aspects of BRCA1 function. Moreover, because the HDR and tumor suppression activities of BRCA1 are dependent on the phospho-recognition property of its BRCT sequences (Shakya et al., 2011), these results suggest that the interactions of BRCA1 with one or more of its other BRCT phospho-ligands are critical for these functions.

**Results**

**The CtipS326A mutation ablates the Brca1-Ctip interaction in mouse cells**

In human cells, the phospho-dependent interaction between BRCA1 and CtIP can be disrupted by an alanine substitution of the relevant CtIP phosphorylation site (S327A; Yu and Chen, 2004). Therefore, we introduced the corresponding mutation (S326A) into the Ctip gene of mouse embryonic stem (ES) cells using either of two targeting constructs. The CtipS326A-geo construct contains the S326A mutation in exon 11 and a loxP-flanked PGK promoter-driven neomycin gene cassette in the adjacent upstream intron (Fig. 1 B). An analogous CtipS326A-hyg construct was generated by replacing the neomycin cassette in intron 10 with a loxP-flanked hygromycin gene cassette. CtipS326A-/- ES cells, which are heterozygous for a Ctip326A-null allele (Ctip-; unpublished data; see Materials and methods), were then electroporated with the CtipS326A-hyg targeting construct, and Southern analysis was used to identify hygromycin-resistant colonies that had undergone homologous recombination at the Ctip allele to yield CtipS326A-hyg/-- ES subclones (Fig. 1 E).
Figure 1. Design of the mutant Ctip\(S326A\) allele and identification of heterozygous ES cells with the Ctip\(S326A\)-hyg or Ctip\(S326A\)-neo knock-in alleles. The wild-type Ctip locus encompassing exons 9–14 is shown (A), along with the Ctip\(S326A\)-neo targeting vector (B), and maps of the Ctip locus after homologous recombination (C) and cre-mediated recombination (D). For the targeting vector, a neomycin expression cassette flanked by lox\(P\) signals (closed triangles) was inserted into the HpaI site of intron 10, whereas the Ctip\(S326A\) mutation (asterisk) and an AgeI restriction site were introduced into exon 11. An HSV thymidine kinase (HSV-TK) gene cassette was included in the targeting vector for negative selection. The wavy line represents plasmid sequences of the targeting vector. Relevant restriction enzyme sites are: PvuII (P), EcoRI (E), HpaI (H), and AgeI (A). The Ctip probe used for Southern analysis and the sizes of the PvuII fragments recognized by the probe are shown. An analogous Ctip\(S326A\)-hyg targeting construct was prepared by replacing the lox\(P\)-flanked neomycin resistance cassette in intron 10 with a lox\(P\)-flanked hygromycin selection marker (note: the hygromycin cassette lacks a PvuII restriction site). To identify heterozygous ES cells with the Ctip\(S326A\)-hyg or Ctip\(S326A\)-neo knock-in alleles, Southern analysis of PvuII-digested genomic DNA with a 5' flanking probe (A) was used to screen (E) hygromycin-resistant Ctip\(+/−\) ES cell subclones targeted with the Ctip\(S326A\)-hyg construct and (F) neomycin-resistant 129/Sv ES cell subclones targeted with the Ctip\(S326A\)-neo construct. The 7.9-kb PvuII germine fragment is converted into a 10.4-kb fragment in properly targeted Ctip\(S326A\)-hyg/+ ES subclones (lanes 2, 4, and 7; panel E) or a 5.8-kb fragment in properly targeted Ctip\(S326A\)-neo/+ ES subclones (lanes 1, 2, and 11; panel F).
To excise the hygromycin gene cassette from the knock-in allele, CtipS326A→byg−/− cells were infected with an adenovirus expressing Cre recombinase and properly recombined CtipS326A→/− ES clones (Fig. 1 D) were identified by Southern analysis and confirmed by nucleotide sequencing. The CtipS326A→− and CtipS326A→− clones, which represent isogenic ES cell lines expressing either wild-type or S326A mutant Ctip, were then used to study the DSB repair functions of Ctip (e.g., see next section).

To produce isogenic mouse embryonic fibroblast (MEF) lines expressing either wild-type or S326A mutant Ctip, 129/Sv ES cells were electroporated with the CtipS326A→− targeted transgene driven by the mouse Rosa26 gene promoter (RosaCre). Significantly, when heterozygous CtipS326A/+ mice were intercrossed, CtipS326A/+ mice were born at the expected (25%) Mendelian ratio, indicating that the S326A mutation does not affect embryonic development. Thus, unlike animals homozygous for either a Brca1- or Ctip-null allele, which undergo embryonic lethality before gastrulation (precluding the generation of MEFs; Liu et al., 1996; Hakem et al., 1997; Ludwig et al., 1997; Chen et al., 2005), CtipS326A/S326A mice are viable. Isogenic primary CtipS326A/+ and CtipS326A/S326A MEFs were prepared from day E13.5 embryos, and immortalized MEF lines were established by transfection with simian virus 40 large T antigen.

The mutant Ctip polypeptide of CtipS326A/S326A MEFs is readily detected by immunoblot analysis (see Figs. 2 A and 6 C), although its steady-state levels appear to be slightly but consistently reduced relative to wild-type Ctip. To ascertain whether S326A mutant Ctip interacts with mouse Brca1, nuclear extracts of Ctip−/−, CtipS326A/+ , and CtipS326A/S326A MEFs were immunoprecipitated with a mouse Brca1-specific antiserum (B1) or the corresponding preimmune serum (Pre) and immunoblotted with an antibody specific for α-tubulin, Brca1, and Ctip. A nonspecific band in the Brca1 immunoblot is designated as “ns.” (B) To evaluate the Brca1–Ctip interaction, the extracts were immunoprecipitated with mouse Brca1-specific antisera (B1) or the corresponding preimmune serum (Pre) and immunoblotted with Brca1-specific monoclonal antibodies. As shown, Ctip was coimmunoprecipitated with Brca1 from Ctip−/− and CtipS326A/+ cells, but not CtipS326A/S326A cells. Note: the amount of nuclear extract used for immunoblotting (A) represents 6.25% of the total extract used for coimmunoprecipitation analysis (B).

Cells expressing the CtipS326A mutant show limited sensitivity to genotoxic agents

Brca1S1598F/I598F cells, which harbor a missense mutation (S1598F) that ablates the BRCT phospho-recognition activity of Brca1, are hypersensitive to the DNA cross-linking agent mitomycin C (MMC; Shaky et al., 2011). This suggests that cellular resistance to MMC is dependent on the interaction of BRCA1 with one or more of its BRCT phospho- ligands. To determine whether the BRCA1–Ctip interaction is required for MMC resistance, isogenic Ctip−/− and CtipS326A−/− ES subclones were evaluated in a clonogenicity assay in parallel with Brca1S1598F/I598F ES cells, which express an internally deleted Brca1 polypeptide that renders cells hypersensitive to MMC (Moyahan et al., 2001a). As shown in Fig. 3, the MMC survival curves of CtipS326A−/− cells, unlike Brca1S1598F/I598F cells, overlap with those of the Ctip−/− and Brca1−/− control cells.
require the interaction of BRCA1 with one or more of its BRCT phospho-ligands (Shakya et al., 2011). To ascertain the role of the BRCA1–CtIP interaction, we examined metaphase spreads of early passage 
\text{Ctip}^{+/+}\text{ and } \text{Ctip}^{S326A/S326A} primary MEF subclones. As shown in Fig. 5 A and Table 1, \text{Ctip}^{S326A/S326A} MEFs displayed low levels of spontaneous chromosomal rearrangements, comparable to those of wild-type \text{Ctip}^{+/+} cells. Moreover, aneuploidy was not observed in \text{Ctip}^{S326A/S326A} cells, which contained on average the expected number of 40 mouse chromosomes. In addition, when subjected to DNA damage by MMC treatment, \text{Ctip}^{S326A/S326A} cells acquired cytogenetic defects to the same extent as control \text{Ctip}^{+/+} MEFs (Fig. 5 B; Table 1). Thus, the Brca1–Ctip interaction is dispensable for suppression of spontaneous and genotoxic-induced chromosomal instability.

The Brca1–Ctip interaction is not required for assembly of Rad51 or RPA nuclear foci
In cells treated with ionizing radiation (IR), Rad51 polypeptides accumulate at sites of DNA damage to form IR-induced foci (IRIFs) that can be visualized by immunofluorescent microscopy. These structures are thought to represent the recruitment of Rad51 polypeptides to sites of DSBs and formation of the ssDNA–Rad51 nucleofilaments necessary for HDR. Because the assembly of Rad51 IRIFs is dependent on the BRCT phospho-recognition property of BRCA1 (Shakya et al., 2011), we examined whether the Brca1–Ctip interaction is also required for this process. As expected, formation of Rad51-staining IRIFs was markedly reduced in Brca1^{S1598F/S1598F} MEFs (Fig. 6 A and Fig. S4). In contrast, assembly of Rad51 IRIFs

Thus, MMC resistance is dependent on the interaction of BRCA1 with one or more of its BRCT phospho-ligands (Shakya et al., 2011), but not solely on its interaction with CtIP.

Ctip-depleted cells are hypersensitive to both the topoisomerase I inhibitor camptothecin (CPT) and the topoisomerase II inhibitor etoposide (ETO; Sartori et al., 2007). By stabilizing their respective Topo cleavage complexes, these agents can block DNA replication and elicit DSB formation. To study the function of the BRCA1–Ctip interaction, Nakamura et al. (2010) generated chicken DT40 cells (\text{Ctip}^{S332A/S332A}) that express a CtIP protein lacking the phosphorylation site (S332) required for its interaction with BRCA1. Interestingly, although these cells were proficient for DSB repair by HDR, they displayed hypersensitivity to both CPT and ETO, suggesting a specific role for the BRCA1–Ctip interaction in processing DSB ends that possess covalently bound polypeptides (Nakamura et al., 2010). To assess the requirement for this interaction in mammalian cells, clonogenic assays were conducted with \text{Ctip}^{+/+} and \text{Ctip}^{S326A/S326A} ES clones. As shown in Fig. 4, A and B, \text{Ctip}^{S326A/S326A} cells displayed modest but reproducible sensitivity to both CPT and ETO relative to the isogenic \text{Ctip}^{+/+} control cells. In contrast, Brca1^{Δ223–763/Δ223–763} cells showed significant hypersensitivity to CPT (Fig. 4 A) and modest sensitivity to ETO (Fig. 4 B). These results suggest that the Brca1–Ctip interaction is required for some, but not all, of the cellular resistance mediated by Brca1 in response to the topoisomerase inhibitors CPT and ETO.

\text{Ctip}^{S326A} cells maintain chromosomal stability at normal rates
Because chromosomal rearrangements arise frequently in Brca1^{S1598F/S1598F} cells, chromosomal stability is likely to be compromised in these cells. As shown in Fig. 3, \text{Ctip}^{S326A/S326A} ES cells are resistant to MMC-induced genotoxic stress. Isogenic \text{Ctip}^{+/+} and \text{Ctip}^{S326A/S326A} ES cells were examined for mitomycin C (MMC) sensitivity in clonogenic survival assays, together with ES cells homozygous for the hypomorphic Brca1^{Δ223–763} mutation (Brca1^{Δ223–763/Δ223–763}) and control ES cells (Brca1^{+/+}). Cells were treated with various concentrations of MMC for 4 h, allowed to recover for 7–9 d, and surviving colonies were stained with Crystal violet. Survival is calculated as a percentage of colonies in the mock-treated plates. Each subclone was tested in triplicate, and the error bars represent the SEM of survival for each subclone.
Brca1–Ctip interaction is not essential for the HDR pathway of DSB repair in mammalian cells

Previous studies have established that DSB repair by the HDR pathway is dependent on both BRCA1 and CtIP (Moynahan et al., 1999, 2001a; Sartori et al., 2007; Bennardo et al., 2008; Chen et al., 2008). Therefore, to determine whether the BRCA1–CtIP interaction is also required we measured HDR at a defined chromosomal break using an integrated DR-GFP recombination substrate (Pierce et al., 2001). The DR-GFP substrate consists of two defective GFP genes: SceGFP, which contains the cleavage site for the I-SceI endonuclease; and iGFP, which lacks the N- and C-terminal coding sequences of GFP (Fig. 7 A). The DR-GFP substrate was integrated into the chromosome of cells transfected with an expression plasmid for a wild-type or mutant CtIP polypeptide. The DR-GFP substrate was then activated by transiently transfecting cells with an expression plasmid for I-SceI endonuclease. Subsequently, the DR-GFP substrate was analyzed by fluorescence microscopy to determine the percentage of cells that had successfully repaired the DSB by HDR.

Upon DNA resection of a DSB, the nascent ssDNA tail is initially coated with the RPA heterotrimer to form an ssDNA–RPA filament which can be observed cytologically by the appearance of nuclear foci that stain with RPA-specific antibodies or biochemically by hyperphosphorylation of the RPA2 subunit. Therefore, to examine whether the BRCA1–CtIP interaction is required for DNA resection, we compared the assembly of damage-induced ssDNA–RPA filaments in isogenic cells that express either wild-type or S326A mutant CtIP. Of note, similar levels of IR-induced RPA foci (Fig. 6 B and Fig. S5) and camptothecin-induced RPA2 hyperphosphorylation (Fig. 6 C) were observed in Ctip+/− and Ctip S326A−/− MEFs (Fig. 6 B and Fig. S5). Thus, the BRCT phospho-recognition property of BRCA1 and, more particularly, the BRCA1–CtIP interaction are dispensable for resection of damage-induced DSB ends in mammalian cells.

The Brca1–Ctip interaction is not essential for the HDR pathway of DSB repair in mammalian cells

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I-SceI expression triggers cleavage of SceGFP, resulting in a chromosomal DSB at the I-SceI site. Repair of this DSB by HDR using iGFP as a template generates a functional GFP gene, such that the frequency of HDR can be quantified as the percentage of GFP-positive cells using flow cytometry (Pierce et al., 2001).

To measure HDR of DSBs at a defined chromosomal site, Ctip+ and CtipS326A/− ES cells were electroporated with p59xDR-GFP6, a DNA construct that contains the DR-GFP recombination substrate and a promoterless hygromycin-resistance marker flanked by targeting arms comprised of mouse Pim1 genomic DNA (Moynahan et al., 2001b). After hygromycin selection, drug-resistant colonies were examined by Southern analysis to identify ES subclones that possess the DR-GFP substrate at an identical position within the Pim1 locus. To measure HDR of an induced chromosomal DSB, the Ctip+ and CtipS326A/− DR-GFP subclones were evaluated for the appearance of GFP-positive cells after transient transfection with an I-SceI expression vector. In the absence of I-SceI, GFP-positive cells were seldom detected (<0.02%) in cells of either Ctip genotype (Fig. 7 B), indicating that spontaneous intrachromosomal gene conversion is rare. As expected, I-SceI expression induced HDR of the DR-GFP substrate in control Ctip+ ES cells, as reflected by the increased percentage of GFP-positive cells (2.4–2.74%; Fig. 7 B). Surprisingly, I-SceI expression induced similar proportions of CtipS326A/− GFP-positive cells (2.13–2.26%; Fig. 7 B), indicating that the Brca1–Ctip interaction is not essential for proficient HDR. Consistent with previous reports (Moynahan et al., 1999, 2001a; Snouwaert et al., 1999), HDR efficiency was significantly reduced (0.64%) in Brca1 mutant ES cells (Brca1S223–763/Δ223–763) with the DR-GFP substrate integrated at the same position of the Pim1 locus (Fig. 7 B). Thus, although the BRCT phospho-recognition activity of Brca1 is required for HDR (Shakya et al., 2011), the Brca1–Ctip interaction appears to be dispensable for DSB repair by this pathway.

The Brca1–Ctip interaction is also dispensable for DSB repair by MMEJ and single-strand annealing

Unlike HDR, Ku-dependent NHEJ ligates DSB ends without a requirement for extensive sequence homology (Symington and Gautier, 2011). In contrast to this classical pathway of NHEJ, microhomology-mediated end joining (MMEJ) employs short sequence homologies to align broken DNA ends before ligation and, as such, is dependent on DNA resection to expose microhomologies within the ssDNA overhangs (Nussenzeew and Nussenzeew, 2007; McVey and Lee, 2008). To examine whether the BRCA1–CtIP interaction is required for MMEJ, we electroporated isogenic Ctip+ and CtipS326A/− ES cells with pim-EJ2-GFP-hyg, a targeting vector that contains the EJ2-GFP recombination reporter, a hygromycin resistance cassette, and genomic sequences for targeting the Pim1 locus (Bennardo et al., 2008). The EJ2-GFP reporter consists of an N-terminal tag fused to GFP, which is disrupted by an 8-nucleotide microhomology repeat that flanks an I-SceI site and stop codons in all three reading frames (Fig. 8 A). If MMEJ occurs by annealing of the microhomology repeats, the intervening 35-nucleotide sequence is deleted, the coding frame between the N-terminal tag and GFP is restored, and a functional GFP gene is reconstructed (Bennardo et al., 2008). After electroporation with the pim-EJ2-GFP-hyg targeting vector, Ctip+ and CtipS326A/− ES cells were selected with hygromycin and DNA was prepared from the surviving colonies. Southern analysis revealed proper homologous integration of the EJ2-GFP reporter into the mouse Pim1 locus of several Ctip+ and CtipS326A/− ES subclones. To measure MMEJ repair of an I-SceI–induced chromosomal break, Ctip+ EJ2-GFP and CtipS326A/− EJ2-GFP subclones were transiently transfected with an I-SceI expression vector and evaluated for the appearance of GFP-positive cells by flow cytometry. As expected, very few GFP-positive cells were detected (<0.05%) in ES subclones transfected with an empty expression vector (Fig. 8 B). However, after I-SceI expression, the proportions of GFP-positive cells were increased to a comparable extent in both the Ctip+ EJ2-GFP (0.64–0.71%)
Table 1. Spontaneous and induced chromosomal aberrations in primary MEFs with different Ctip genotypes

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<tr>
<th>Genotype</th>
<th>Metaphases analyzed</th>
<th>MMC treatment</th>
<th>Metaphase aberrations</th>
<th>Chr/Cht breaks and gaps</th>
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The percentage of metaphases containing one or more aberrations and a breakdown of aberration type is shown for each primary MEF cell line in both the absence (−) and presence (+) of MMC. Cht, chromatid; Chr, chromosome; MMC, mitomycin C.

and CtipS326A−/− EJ2-GFP (0.62–0.64%) subclones (Fig. 8 B). Thus, as with the resection-dependent HDR pathway, the Brca1–Ctip interaction appears to be dispensable for MMEJ repair of chromosomal DSBs.

Single-strand annealing (SSA) is a mutagenic mode of DSB repair that shares some features with MMEJ (Bennardo et al., 2008; McVey and Lee, 2008). In SSA, annealing occurs between long (>30 nucleotides) direct repeats that flank the DSB, allowing for ligation of the ends and deletion of the intervening sequences. Although the late stages of repair by SSA and MMEJ are distinct, both pathways require CtIP-mediated DNA resection to expose complementary sequences within the ssDNA overhangs (Bennardo et al., 2008; McVey and Lee, 2008). Previous studies have established that efficient DSB repair by SSA is dependent on both BRCA1 and CtIP (Stark et al., 2004; Bennardo et al., 2008). Therefore, to ascertain whether the BRCA1–CtIP interaction is also required for SSA, isogenic Ctip−/− and CtipS326A−/ES cells were electroporated with the hprtSAGFP targeting construct (Stark et al., 2004), and subclones that carry the SA-GFP recombination reporter integrated into the Hprt locus were derived. The SA-GFP reporter, which contains a restriction site for I-SceI cleavage, generates a functional GFP gene when repaired by SSA (Fig. 8 C; Stark et al., 2004). Therefore, to measure the efficiency of SSA in Ctip−/− and CtipS326A−/ES subclones with an integrated SA-GFP substrate, these cells were transiently transfected with an I-SceI expression vector. As shown in Fig. 8 D, I-SceI expression elicited comparable levels of GFP-positive cells in both the Ctip−/−/SA-GFP (0.76–1.13%) and CtipS326A−/SA-GFP (1.07–1.40%) subclones. Thus, although Brca1 and Ctip are each essential for SSA (Stark et al., 2004; Bennardo et al., 2008), the Brca1–Ctip interaction is not required for this mode of DSB repair.

Tumor suppression is not dependent on the Brca1–Ctip interaction

Homozygous Brca1S1598F/S1598F mice, which express a mutant Brca1 protein defective for BRCT phospho-recognition, are prone to tumor development (Shakya et al., 2011). This observation implies that the interaction of BRCA1 with one or more of its BRCT phospho-ligands is required for tumor suppression. To assess the role of the Brca1–Ctip interaction in tumor suppression, we monitored cohorts of 45 control (Ctip+/+) and 40 mutant (CtipS326A/S326A, CtipS326A-neo/S326A-neo, or CtipS326A-neo−/−) mice for tumor development over a 24-month observation period. As shown in Fig. 9, a few mutant mice developed tumors at a very advanced age, with kinetics (frequency and latency) statistically indistinguishable from that of the control cohort (750 days; P = 0.2099), but significantly delayed relative to the tumor-prone Brca1S1598F/S1598F mice (P < 0.0001). Thus, although tumor suppression by BRCA1 is dependent on the phospho-recognition potential of its BRCT repeats (Shakya et al., 2011), solely disrupting the Brca1–Ctip interaction does not predispose mice to tumor formation.

Discussion

The BRCA1 tumor suppressor has emerged as a central player in the cellular response to DNA damage (Huen et al., 2010; Moynahan and Jasim, 2010; Li and Greenberg, 2012; Roy et al., 2012). Of particular interest, BRCA1 is required for homology-directed repair (HDR), a relatively error-free pathway for repair of DSBs. Because HDR defects can lead to both chromosome rearrangements and aneuploidy, loss of HDR function may be a primary source of the genomic instability that is characteristic of BRCA1 mutant cells (Huen et al., 2010; Moynahan and Jasim, 2010; Li and Greenberg, 2012; Roy et al., 2012). Interestingly, the BRCA2 tumor suppressor has also been implicated in HDR (Moynahan et al., 2001b) and ascribed a specific biochemical function in formation of the ssDNA–Rad51 nucleoprotein filament, an essential HDR intermediate (Yang et al., 2005). Thus, HDR deficiency may be a common determinant of breast cancer susceptibility in both BRCA1 and BRCA2 mutation carriers (Huen et al., 2010; Moynahan and Jasim, 2010; Li and Greenberg, 2012; Roy et al., 2012). Although the precise biochemical role of BRCA1 in HDR remains unclear, the phospho-recognition property of its BRCT repeats is required for both HDR and tumor suppression (Shakya et al., 2011). In this regard, it is noteworthy that each of the three phospho-ligands known to form in vivo protein complexes with BRCA1 (i.e., Abraxas/CCDC98, BACH1/FancJ/BRIP1, and CtIP) has also been implicated in DSB repair by the HDR pathway (Huen et al., 2010; Moynahan and Jasim, 2010; Li and Greenberg, 2012; Roy et al., 2012).

CtIP collaborates with the MRN (Mre11–Rad50–Nbs1) complex to initiate resection of DSB ends and formation of the
Figure 6. CtipS326A/S326A MEFs are proficient for assembly of Rad51 and RPA nuclear foci in response to DNA damage. (A and B) Ctip+/+ and CtipS326A/S326A MEFs were exposed to IR (10 Gy) and IRIF formation was assessed 1 h later by immunostaining with rabbit antisera specific for Rad51 (A) or Thr21-phosphorylated RPA2 (B). Cells containing 10 or more distinct Rad51- or phosphorylated RPA2-staining nuclear foci were counted in at least 500 nuclei of two independent MEF lines for each genotype, and the error bars represent SEM. IR treatment strongly induced the number of Rad51 foci in Ctip+/+ and CtipS326A/S326A MEFs, but not in Brca1S1598F/S1598F MEFs, which are known to have reduced IRIF assembly of Rad51 (Shakya et al., 2011). IR treatment also strongly induced the number of Thr21-phosphorylated RPA2 IRIFs in Ctip+/+ and CtipS326A/S326A MEFs, as well as in Brca1S1598F/S1598F MEFs. (C) Independent clones of Ctip+/+ and CtipS326A/S326A MEFs were cultured in the presence or absence of 1.0 µM camptothecin (CPT) and harvested 1 h later. Total cell extracts of each culture were then fractionated by SDS-PAGE and immunoblotted with antibodies specific for Ctip, Ser4/Ser8-phosphorylated RPA2, total RPA2, or α-tubulin.
3′-ssDNA overhangs required for HDR and MMEJ (Sartori et al., 2007; Bennardo et al., 2008; Chen et al., 2008). Because BRCA1 has been implicated in these same repair pathways, it is conceivable that BRCA1–CtIP interaction, is involved in the DNA resection functions of CtIP. This notion is very attractive, as it could provide a biochemical mechanism to explain how BRCA1 promotes the HDR pathway of DSB repair. A requirement for BRCA1 in CtIP-mediated resection would also be consistent with genetic data that place BRCA1 upstream of BRCA2 in the HDR pathway (Stark et al., 2004). Moreover, BRCA1, CtIP, and MRN are known to form a discrete protein complex in mammalian cells that could potentially mediate the resection activities ascribed to CtIP and MRN (Greenberg et al., 2006; Chen et al., 2008).

At present, however, published data regarding the role of BRCA1 in DNA resection are contradictory. On the one hand, Schlegel et al. (2006) assessed ssDNA formation in BrdU-labeled cells cytologically by the appearance of nuclear foci that stain with BrdU-specific antibodies. Using this approach, they showed that ssDNA focus formation in response to ionizing radiation (IR) is abolished by siRNA-mediated depletion of BRCA1 (Schlegel et al., 2006). Moreover, on the basis of BRCA1 reconstitution experiments in HCC1937 cells, a human breast tumor line that expresses a truncated BRCA1 polypeptide lacking its C-terminal BRCT motif, they concluded that the ability of BRCA1 to promote end resection is independent of its BRCT sequences. On the other hand, Chen et al. (2008) found that IR-induced assembly of nuclear ssDNA/RPA foci, detectable by staining with RPA-specific antibodies, is impaired in HCC1937 cells, but not in HCC1937 cells reconstituted with wild-type BRCA1. Thus, they also concluded that BRCA1 promotes end resection, but that it does so in a manner dependent on its BRCT sequences (Chen et al., 2008). Finally, Escribano-Díaz et al. (2013) described a modest reduction in IR-induced RPA focus formation upon siRNA depletion of BRCA1 in U2OS cells, but Zhao et al. (2007) observed no effect in HeLa cells.

The role of the BRCA1–CtIP interaction itself in DNA resection and DSB repair is also controversial. To address this issue, Yun and Hiom (2009) generated CtIP-null cells (CtIP−/−) from the chicken B cell tumor line DT40. The efficiency of the major DSB repair pathways was then measured in CtIP-null cells reconstituted with either a wild-type (CtIP+/+ + hCtIP) or S327A mutant (CtIP−/− + hCtIP-S327A) form of human CtIP. Notably, cells expressing mutant CtIP were competent for DSB repair by NHEJ and MMEJ, but displayed marked defects in DNA repair by pathways that entail extensive DNA resection (i.e., HDR and SSA). These striking results implicated BRCA1 in CtIP-mediated DNA resection and suggested a key role for the BRCA1–CtIP interaction in directing the pathway choice for DSB repair (Yun and Hiom, 2009). Subsequently, however, Nakamura et al. (2010) reported that HDR function is proficient in any mouse cells (CtIP−/− + hCtIP-S327A) expressing endogenous CtIP with the corresponding mutation (S332A in chickens). Moreover, Peterson et al. (2011) showed that mutation of the corresponding phosphorylation site in frog xCtIP (S328A) does not affect its ability to stimulate DNA resection in Xenopus cell-free extracts. Therefore, to determine whether the BRCA1–CtIP interaction is required for DNA resection and DSB repair in mammals, we generated isogenic subclones of mouse ES cells that express mouse Ctip with and without the S326A mutation. Our results indicate that the BRCA1–CtIP interaction in mammalian cells is dispensable for the major resection-dependent
pathways of DSB repair, including HDR, MMEJ, and SSA. In accord with these results, Ctip^{S326A/S326A} mouse embryonic fibroblasts displayed normal suppression of spontaneous and genotoxic-induced chromosomal instability as well as normal assembly of IR-induced Rad51 and RPA foci.

Although two independent studies using chicken DT40 cells have generated contradictory conclusions about the function of the BRCA1–CtIP interaction (Yun and Hiom, 2009; Nakamura et al., 2010), our results indicate that, in mammalian cells, this interaction is not required for DNA resection or the resection-dependent modes of DSB repair. As such, our findings concur with those of Nakamura et al. (2010), who observed normal HDR levels in DT40 cells (Ctip^{S326A/S326A}) expressing CtIP polypeptides that fail to bind BRCA1. Interestingly, these cells also displayed marked hypersensitivity to camptothecin (CPT), a topoisomerase 1 (Topo1) inhibitor that ultimately generates 3'-DSB ends covalently linked to Topo1, and to a lesser degree etoposide (ETO or VP16), a topoisomerase 2 (Topo2) inhibitor that yields Topo2-linked 5'-DSB ends. On this basis, Nakamura et al. (2010) proposed that the BRCA1–CtIP interaction promotes the endonucleolytic cleavage of oligonucleotide-bearing covalently bound polypeptides from DSB ends. Although yeast
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Tumor-Proneness of the BRCA1-Ctip Interaction

We observed a very modest, but reproducible, sensitivity of cell-free extracts (Peterson et al., 2011) and that the CtIP orthologue Ctp1 has been implicated in excision of covalently bound Topo2 protein from DSB ends in Schizosaccharomyces pombe (Hartsuiker et al., 2009). We observed a very modest, but reproducible, sensitivity of Ctip^{S326A}/ES cells to camptothecin and, to a lesser extent, etoposide. Although the pattern of drug sensitivities in Ctip^{S326A/}ES cells mirrors that seen in Ctip^{S326A/} chicken DT40 cells, the magnitude of drug sensitivity is much more pronounced in the mutant chicken cells. On one hand, this quantitative difference may simply represent species-specific variation in cellular resistance to the topoisomerase inhibitors. On the other hand, it may reflect the fact that chicken DT40 cells, unlike mouse embryonic stem cells, are fully transformed tumor cells that harbor a variety of tumor-associated genetic lesions, one or more of which might augment the drug sensitivity of its Ctip mutant subclones.

Our results indicate that the Brca1–Ctip interaction is dispensable for Ctip-mediated resection and for the resection-dependent modes of DSB repair, such as HDR, MMEJ, and SSA. These conclusions are supported by biochemical data in cell-free extracts (Peterson et al., 2011) and genetic data in chicken DT40 cells (Nakamura et al., 2010). What, then, are the cellular functions of the evolutionarily conserved Brca1–Ctip interaction? Previous studies have implicated this interaction in activation of the G2/M cell cycle checkpoint (Yu and Chen, 2004) and, more recently, in preventing aberrant recruitment of the RIF1 repair protein to damaged DNA in S/G2 cells (Escriberno-Diaz et al., 2013). Although both of these activities likely facilitate the cellular response to DNA damage, the absence of heightened tumor susceptibility in Ctip^{S326A/S326A} mice suggests that they are not required for tumor suppression.

Although not yet formally proven, the HDR function of BRCA1 is thought to be a critical, if not essential, aspect of its tumor suppression activity. Indeed, a homozygous Brca1 mutation that ablates BRCT phospho-recognition (Brca1^{S1598F/S1598F}) also disrupts HDR and renders mice prone to tumor development (Shakya et al., 2011). This implies that the ability of BRCA1 to bind one or more of its BRCT phospho-ligands is critical for both HDR and tumor suppression. Here we show that tumor susceptibility is not enhanced by a mutation that specifically ablates the Brca1–Ctip interaction in mice (Ctip^{S326A/S326A} and Ctip^{S326A/}). These results suggest that the Brca1–Ctip interaction is dispensable for BRCA1-mediated tumor suppression altogether or that its role in tumor suppression can be compensated by one or more of the other BRCT phospho-ligands.

Materials and methods

The Ctip^{S326A} targeting constructs

The homozygous lines of the Ctip^{S326A/} targeting construct (Fig. 1 B) were derived from a 6-kb EcoRI fragment of genomic DNA, encompassing Ctip exons 10–12, from strain 129-derived E14 TG2a ES cells (Warren et al., 1994). Site-directed mutagenesis of exon 11 was used to (1) replace the natural serine codon (TCT) for residue 326 with an alanine codon (GCA), and (2) convert the sequence TCCGCT into an AgeI restriction site [AGCGGT]. A loxP-flanked PGK promoter-driven neo expression cassette was inserted into a unique Hpal site in intron 10 (Fig. 1 B). A gene cassette encoding herpes simplex virus type 1 thymidine kinase (HSV-TK) was also included in the construct as a negative selection marker. The Ctip^{S326A/+} targeting construct was prepared by replacing the loxP-flanked neo expression cassette of the Ctip^{S326A/} construct with a loxP-flanked hygromycin expression cassette (lacking a pUk site).

Analysis of isogenic ES cell subclones

Embryonic stem (ES) cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM; Cellgro) supplemented with 15% heat-inactivated FBS (HyClone ES Cell Screened; Thermo Fisher Scientific), 2 mM l-glutamine, 1% nonessential amino acids, 100 µg/ml penicillin/streptomycin, 0.1 mM 2-mercaptoethanol, 1.25 µg/ml Plasmocin (InvivoGen), and 1,000 units/ml LIF (Esgro; EMD Millipore) at 37°C in 5% CO2/95% humidity. Unless otherwise noted, ES cells were grown on a layer of mitotically inactive primary embryonic fibroblasts [feeders] in the presence of LIF to maintain their pluripotency. Not-linearized DNA of the Ctip^{S326A/+} targeting construct was introduced by electroporation (30 µg of DNA at 0.8 kV/3 µF) into Ctip^{+/+} ES cells; the functionally null Ctip allele of these cells was generated previously by replacing 1.2 kilobases of Ctip genomic DNA, including part of exon 11 and all of exon 12, with an neo promoter-driven neo expression cassette. After hygromycin selection, drug-resistant ES cell subclones were examined for gene targeting by Southern analysis of PvuII-digested genomic DNA with a 5′ flank probe that spans Ctip exon 9 (Fig. 1 A). Correctly targeted Ctip^{S326A/+}-clones were then infected with Adeno-Cre virus, and DNA from individually picked ES cell colonies were screened by Southern analyses for removal of the loxP-flanked hygromycin cassette and generation of the desired Ctip^{S326A/} subclones (Fig. 1 D). To conduct clonogenic survival assays, ES cells were exposed for 4 h (mitomycin C) or 24 h (camptothecin and etoposide) to various concentrations of the drug, washed twice with 1× PBS, provided fresh media, and allowed to grow at 37°C for 7–9 d. Colonies of surviving cells were then fixed in 10% buffered formalin for 30 min, stained with 0.5% Crystal Violet (Sigma-Aldrich) for 15 min, washed in water three times, and then counted. Survival experiments were performed in triplicates.
Recombination reporter assays
To assess homology-directed repair (HDR), ES subclones containing the DR-GFP reporter integration into the pim1 locus were generated. HDR was analyzed using ES cells transfected with either the pCAGGS-Ctip expression vector (pCAGGS-Ctip/+) or pCAGGS-Ctip expression vector (pCAGGS-Ctip−/−). ES cells were transfected with either Ctip+/+ or Ctip−/−. The next day, ES cells were washed twice with PBS, and the harvested cells were used for Southern analysis for proper integration of the DR-GFP reporter. The insertional reporter vector was integrated into the pim1 locus, and the expression of both the GFP and Ctip protein was detected. The GFP reporter was used to identify the DR-GFP-expressing ES cells, and the expression of Ctip was used to confirm the presence of the DR-GFP vector. The Ctip expression was detected using a mouse monoclonal antibody that recognizes total Ctip (14-1). The GFP reporter was used to identify the DR-GFP-expressing ES cells, and the expression of Ctip was used to confirm the presence of the DR-GFP vector. The Ctip expression was detected using a mouse monoclonal antibody that recognizes total Ctip (14-1). The GFP reporter was used to identify the DR-GFP-expressing ES cells, and the expression of Ctip was used to confirm the presence of the DR-GFP vector. The Ctip expression was detected using a mouse monoclonal antibody that recognizes total Ctip (14-1).

Analysis of isogenic mouse embryonic fibroblasts
Primary Ctip+/-, Ctip−/-, and Ctip+/−/Ctip−/− mouse embryonic fibroblasts (MEFs) were derived from E13.5 embryos and cultured in DMEM (Cellgro) supplemented with 10% heat-inactivated FBS (Tissue Culture Biologicals), 100 µg/ml penicillin/streptomycin, 2 mm l-glutamine, and 1.25 µg/ml Plasmocin (Invivogen) at 37°C in 5% CO2/95% humidity. Immortalized MEFs were then generated by transfecting primary MEFs with the pcDNA3.1 vector containing the Ctip expression construct (pCAGGS-Ctip). Cells were harvested in 1× PBS (Hyclone), and the harvested cells were used for Southern analysis for proper integration of the DR-GFP reporter. The insertional reporter vector was integrated into the pim1 locus, and the expression of both the GFP and Ctip protein was detected. The GFP reporter was used to identify the DR-GFP-expressing ES cells, and the expression of Ctip was used to confirm the presence of the DR-GFP vector. The Ctip expression was detected using a mouse monoclonal antibody that recognizes total Ctip (14-1). The GFP reporter was used to identify the DR-GFP-expressing ES cells, and the expression of Ctip was used to confirm the presence of the DR-GFP vector. The Ctip expression was detected using a mouse monoclonal antibody that recognizes total Ctip (14-1). The GFP reporter was used to identify the DR-GFP-expressing ES cells, and the expression of Ctip was used to confirm the presence of the DR-GFP vector. The Ctip expression was detected using a mouse monoclonal antibody that recognizes total Ctip (14-1).
of nucleotides, fluorescently labeled oligonucleotides [Detection Reagent Red], and Polymerase (Duolink). The cells were then mounted onto a glass slide using Duolink in situ mounting medium with DAPI. Immunostaining analysis and images were acquired at room temperature using a microscope (Axio Imager.Z2; EC Plan-Neofluar 40x/0.75 NA objective lens; Carl Zeiss) equipped with a CoolCube 1 camera [MetaSystems], a motor-controlled stage [MetaSystems], and the Isis and Metafer 4 software packages [MetaSystems]. Green [Alexa Fluor 488] and blue [DAPI] images of the same cells were merged using Image] software [National Institutes of Health] and the final images were prepared in Adobe Photoshop CS5 extended version 12.0.

Online supplemental material

Fig. S1 shows that the in situ association of Brca1 and Ctip is markedly reduced in CtipS326A,S326E MEFs. Fig. S2 shows that the Ctip–S326A poly- peptide interacts with Mre11 and supports Ctk1 phosphorylation. Fig. S3 shows that CtipS326A,S326E MEFs are proficient for assembly of Ctip nuclear foci in response to DNA damage. Fig. S4 shows that CtipS326A,S326E MEFs are proficient for assembly of Rad51 nuclear foci in response to DNA damage. Fig. S5 shows that CtipS326A,S326E MEFs are proficient for assembly of RPA nuclear foci in response to DNA damage. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201302145.DC1. Additional data are available in the JCB DataViewer at http://dx.doi.org/10.1083/jcb.201302145.

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