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Inhibition of homologous recombination by the PCNA-interacting protein PARI

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Summary

Inappropriate homologous recombination (HR) causes genomic instability and cancer. In yeast, the UvrD family helicase Srs2 is recruited to sites of DNA replication by SUMO-modified PCNA, where it acts to restrict HR by disassembling toxic RAD51 nucleofilaments. How human cells control recombination at replication forks is unknown. Here, we report that the protein PARI, containing a UvrD-like helicase domain, is a PCNA interacting partner, required for preservation of genome stability in human and DT40 chicken cells. Using cell-based and biochemical assays, we show that PARI restricts unscheduled recombination by interfering with the formation of RAD51-DNA HR structures. Finally, we show that PARI knockdown suppresses the genomic instability of Fanconi Anemia/BRCA pathway-deficient cells. Thus, we propose that PARI is a long sought-after factor that suppresses inappropriate recombination events at mammalian replication forks.

Introduction

The ability of cells to repair DNA lesions and to correctly propagate their genetic information is essential for all organisms. Due to the pleiotropic effects generated by the accumulation of mutations, DNA repair deficiencies result in tissue degeneration and aging, as well as cellular transformation and carcinogenesis. Cells possess many DNA repair mechanisms, which survey the DNA landscape throughout the cell cycle, searching for DNA lesions and mismatches. To ensure that the genome is protected, these mechanisms can be highly redundant. DNA repair pathways compete with each other to remove certain lesions. Conversely, DNA repair mechanisms are under strict cellular control, to ensure that

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DNA lesions are corrected with the best outcome, considering the type of lesion, the location in the chromatin, and the position during the cell cycle.

Homologous recombination (HR) acts during S-phase and G2 to repair strand breaks using the intact sister chromatid as a repair template (Helleday, 2010; Moynahan and Jasin, 2010). HR is important for maintaining genome stability since in general it employs an error-free mode of repair. In its absence, repair is channeled into more-error prone pathways such as Non-homologous end joining (NHEJ), leading to the accumulation of mutations and rearrangements. Critical HR factors, such as BRCA2 and RAD51C, are tumor suppressors (Meindl et al., 2010; Wooster et al., 1995), and cells from patients with inactivating mutations in these genes display increased genomic instability. HR is also required for the efficient restart of stalled replication forks during S-phase (Budzowska and Kanaar, 2009).

HR repair is initiated by DNA resection at a double strand break (DSB), revealing a single stranded DNA end which is initially coated by the single strand binding protein complex RPA (San Filippo et al., 2008). In a subsequent step, recombination mediator proteins such as BRCA2 and RAD52 catalyze the replacement of RPA with RAD51, resulting in the formation of RAD51 presynaptic nucleofilaments. RAD51, an ATP-hydrolyzing protein, has a high affinity for DNA in its ATP-bound form and is released from DNA following ATP hydrolysis (Petalcorin et al., 2006). BRCA2 recruits RAD51 to resected DNA, and stabilizes the resulting RAD51 nucleofilament by inhibiting RAD51 ATP hydrolysis (Jensen et al., 2010). The RAD51 filament then catalyzes strand invasion into homologous duplex DNA, leading to formation of a displacement loop (D-loop). Following removal of RAD51 by DNA helicases such as HELQ and RAD54 (Solinger et al., 2002; Ward et al., 2010), the D-loop is extended by DNA polymerases (Li et al., 2009; McIlwraith et al., 2005; Moldovan et al., 2010). Finally, processing of HR structures formed by the extended heteroduplex leads to completion of DNA repair.

Due to its essential role in genome maintenance, the HR pathway is under strict control. Inappropriate hyper-recombination is associated with genomic instability and cancer (Martin et al., 2007; Schild and Wiese, 2010). To ensure that HR is restricted to S and G2, the initial, end resection step is regulated by CDK-dependent phosphorylation of the nuclease machinery (Huertas et al., 2008). A number of DNA damage signaling pathways activate HR by recruiting HR factors to DNA lesions. For example, a complicated cascade of protein post-translational modifications, initiated by the ATM and ATR kinases, regulate DSB recruitment of HR proteins (Bekker-Jensen and Mailand, 2010).

Another mechanism implicated in HR activation is the Fanconi Anemia (FA) DNA repair pathway (Moldovan and D'Andrea, 2009). Initially identified through its inactivation in patients with an inherited genetic disorder characterized by severe anemia, developmental defects, and cancer proneness, the FA pathway coordinates the removal of DNA crosslinks, in a complex process involving HR, Nucleotide Excision Repair (NER) and Translesion Synthesis (TLS) factors. In FA cells, HR is reduced (Nakanishi et al., 2005; Smogorzewska et al., 2007). Hypomorphic mutations of the HR factors BRCA2 and PALB2 are responsible for FA subtypes D1 and N, respectively (Howlett et al., 2002; Reid et al., 2007). Most FA genes encode components of a large complex with ubiquitin ligase activity, which, in response to damage during S-phase, mono-ubiquitinates the critical substrates FANCD2 and FANCI. Subsequently, these proteins move to subnuclear foci where they co-localize with other FA factors, such as the helicase FANCJ and FANCD1/BRCA2, as well as with other DNA replication and repair factors (PCNA, RAD51). How the FA pathway coordinates crosslink repair and HR is remains unclear.

HR is also modulated by helicases that control the RAD51 nucleofilament. In yeast, the UvrD-domain helicase Srs2 inhibits inappropriate HR by removing RAD51 from single stranded DNA, thus reversing the HR reaction at an early step (Krejci et al., 2003). Srs2 binds RAD51 directly and stimulates its ATP hydrolysis activity, leading to the release of RAD51 from DNA (Antony et al., 2009). In this manner, RAD51 nucleofilaments are efficiently disassembled. In higher eukaryotes a clear Srs2 homolog has not been described. Instead, the RAD3-type helicase RTEL1 was shown to remove RAD51 from D-loops in worm and human cells, thereby inhibiting HR by acting at a different, later step than yeast Srs2 in the HR reaction (Barber et al., 2008).

Srs2 is recruited to the replication fork during S-phase, where it blocks unwanted recombination occurring during DNA replication (Papouli et al., 2005; Pfander et al., 2005). This recruitment requires the interaction of Srs2 with PCNA, an essential replication fork component (Moldovan et al., 2007). PCNA post-translational modifications by ubiquitin and SUMO are important decision makers at the replication fork. PCNA mono and K63-linked multiubiquitination events are induced by DNA damage, and control lesion bypass pathways at stalled replication forks. In contrast, PCNA SUMOylation appears constitutively during S-phase, and is required for recruitment of Srs2 via a PCNA-SUMO interacting domain in its C-terminus. To our knowledge, PCNA SUMOylation has not been described in mammalian cells so far.

In an effort to identify novel HR regulators in mammalian cells, we searched the human proteome for factors with similar domain organization as yeast Srs2. Here, we show that the protein C12orf48, containing an UvrD helicase-related domain, is a PCNA-interacting factor. Using cell-based and biochemical assays, we demonstrate that C12orf48 is an anti-recombinase in human and chicken DT40 cells. Thus, we named this factor PCNA-Associated Recombination Inhibitor (PARI). Finally, we show that PARI downregulation improves homologous recombination and genomic stability in HR-deficient Fanconi Anemia/BRCA pathway-inactivated cancer cells.

Results

PARI interacts with PCNA and RAD51

The control of homologous recombination is essential for genomic stability and correct DNA repair. Using a bioinformatic approach, we identified a UvrD helicase-related domain in the sequence of the human protein C12orf48 (Supplemental Figure S1A). Phylogenetic tree analysis of UvrD families showed that C12orf48/PARI closely clusters with family members from multiple species (Supplemental Figure S1B). Moreover, we found that PARI contains a PCNA interacting motif known as PIP-box (Moldovan et al., 2007), as well as a SUMO-interacting motif -SIM (Hecker et al., 2006) (Supplemental Figure S1C). Thus, the domain organization of PARI has similarities with that of yeast Srs2 (Figure 1A).

To investigate the protein interaction properties of PARI, we cloned the full-length cDNA from HeLa cells. Using GST pulldowns, we demonstrated that full-length PARI protein interacts with PCNA from extracts of HeLa cells prepared under native conditions (Figure 1B). Next, we employed truncations of PARI, with or without the PIP-box (depicted in Figure 1A). The PIP-box was required for the interaction of PARI with PCNA (Figure 1C). We also expressed full-length or various truncations of PARI, with a Myc tag, in 293T cells and performed immunoprecipitation experiments to analyze PCNA binding. PCNA coimmunoprecipitated with PARI; deletion of the C-terminal PIP-box abolished this interaction (Figure 1D, E).

PCNA SUMOylation has been reported in yeast, *Xenopus* and chicken cells, but not in mammalian cells (Moldovan et al., 2007). Upon overexpression of His-tagged SUMO1 followed by denaturing Ni-NTA pulldown, we could detect SUMO1 modified PCNA in human 293T cells (Supplemental Figure S1E, F). Moreover, we could conjugate PCNA with SUMO1 *in vitro* (Supplemental Figure S1G–I). Since the modification is present at very low levels, we could not detect PARI binding to SUMOylated PCNA in cell extracts. Instead, we expressed and purified from *E. coli* a linear PCNA-SUMO1 fusion protein. In GST pulldowns PARI bound better to PCNA-SUMO1 than to PCNA alone; deletion of the PARI SIM reduced this preferential binding (Figure 1F, Supplemental Figure S1J).

Sequence analysis did not reveal an obvious RAD51-binding domain in PARI. Nevertheless, we tested whether PARI interacts with RAD51. Using GST pulldowns, we observed that recombinant PARI interacts with RAD51 from native HeLa extracts. Moreover, GST-tagged PARI also pulled-down recombinant human RAD51 expressed and purified from bacteria (Figure 1G). We also examined this interaction using RAD51 GST pulldowns. GST-tagged human RAD51, obtained from bacteria, precipitated Myc-tagged PARI expressed in HeLa cells, as well as recombinant Flag-tagged PARI obtained from baculovirus-infected insect cells (Figure 1H). Using GST-RAD51 pulldowns with PARI truncations expressed in human cells, we found that RAD51 interacts with a domain spanning aminoacids 411–487 of PARI (Figure 1I). Taken together, these results indicate that the UvrD helicase domain-containing protein PARI is a PCNA and RAD51 interacting factor.

Human PARI is required for genomic stability and suppression of recombination

In order to investigate the cellular functions of PARI, we knocked-down its expression using RNA interference in human cells (Supplemental Figure S2A). An antibody raised against PARI failed to detect the endogenous protein; however, the antibody recognized exogenously-expressed PARI (Figure 2A). siRNA-treated 293T exhibited a significant increase in mitomycin C (MMC)-induced chromosomal aberrations visualized in metaphase spreads, including radial chromosomes, chromatid breaks, and chromosome fusions (Figure 2B, C, Supplemental Figure S2B). Moreover, knockdown of PARI in HeLa cells conferred cellular hypersensitivity to MMC (Figure 2D, E). These results demonstrate that PARI is essential for genome stability.

We next investigated the efficiency of HR in human cells depleted of PARI. We used a cell-based assay, which measures the efficiency of direct repeat recombination of two GFP alleles, in U2OS cells (Nakanishi et al., 2005). Knockdown of PARI resulted in a significant increase in HR efficiency, similar to that obtained by depleting the known anti-recombinase RTEL1 (Figure 2F). As expected, knockdown of BRCA2 abolished HR in this assay. Moreover, we found that depletion of PARI from HeLa cells caused a significant increase in basal and IR-induced RAD51 foci (Supplemental Figure S2C, D). These findings suggest that human PARI is a recombination inhibitor, and suggest that in its absence, the HR pathway becomes hyperactive and potentially deleterious.

Hyper-recombination phenotype of PARI-knockout avian DT40 cells

In order to confirm the aforementioned results in a different genetic system, we employed the chicken B-cell lymphocyte cell line DT40. In order to inactivate chicken PARI, we deleted the first two exons of both alleles on chromosome 1 (Figure 3A). In this way, we obtained a DT40 cell line with complete knockout of PARI, as shown by Southern blot and RT-PCR experiments (Figure 3B, C). We used this clone to further examine the role of PARI in HR suppression.

First, we used a DT40-adapted assay to measure direct repeat recombination between two neomycin alleles (Hochegger et al., 2006). Corroborating the situation in human cells, we observed that loss of PARI results in a significant increase in the frequency of gene conversion (Figure 3D). These data further reinforce the idea that PARI functions to keep homologous recombination in check.

Next, we examined the phenotype of the PARI gene-targeted DT40 cells in response to camptothecin (CPT) treatment, which is known to produce an S-phase specific substrate for HR repair. CPT crosslinks topoisomerase 1 to DNA; when the replication machinery encounters these toxic structures in S-phase, it converts them into DSBs (Liu et al., 2000). In DT40 cells, the HR pathway is required for the repair of the majority of CPT-induced lesions (Adachi et al., 2004). We first measured sister chromatid exchanges (SCEs), which result from homologous recombination activity (Sonoda et al., 1999). *PARI*−/− DT40 cells exhibited increased CPT-induced SCEs (Figure 3E and Supplemental Figure S3A, B), suggesting that S-phase recombination is hyperactive. Furthermore, in *PARI*−/− cells, RAD51 foci were significantly increased under basal and DNA-damaging conditions, including CPT (Figure 3F, G) and IR (Figure 3H, I and Supplemental Figure S3C, D) treatment, again suggesting that HR is hyperactive. Importantly, this phenotype was corrected by introducing Myc-tagged human PARI into the knockout cells (Figure 3H, I and Figure 4A), demonstrating a specific effect. Taken together, these results establish that PARI is a specific homologous recombination repressor in higher eukaryotes.

PCNA is essential for PARI functions

In order to investigate the function of the PARI interaction with PCNA, we transfected the *PARI*−/− DT40 cells with the cDNA encoding either human wildtype PARI protein, or versions of PARI lacking the PIP-box or the SIM (Figure 4A). *PARI*−/− cells were hypersensitive to camptothecin (Figure 4B) and wildtype human PARI was able to correct this phenotype. Interestingly, both PIP and SIM-deficient human PARI variants failed to rescue the *PARI*−/− sensitivity to camptothecin (Figure 4B). The PCNA interaction is therefore essential for PARI to regulate HR in response to camptothecin.

Most PIP-box containing proteins require PCNA interaction for correct localization and recruitment to their sites of action on chromatin during S-phase (Moldovan et al., 2007). We observed that PARI expressed in HeLa cells is preferentially localized to chromatin in response to S-phase arrest, but not in mitosis (Supplemental Figure S4A, B). We reasoned that PARI might require PCNA interaction for this localization. Extracts from HeLa cells expressing either wildtype or PCNA interaction-deficient PARI, and treated with hydroxyurea, were fractionated into chromatin and soluble proteins. Compared to wildtype PARI, the chromatin localization of the PCNA interaction-deficient mutant was significantly impaired (Figure 4C, D). These data demonstrate that the PCNA interaction targets PARI to chromatin, which is essential for PARI activity.

In vitro activities of PARI

Although PARI possesses a UvrD-like region, we failed to detect the presence of Walker A or B domains, required for ATP binding and hydrolysis, suggesting that PARI might not be an active ATPase. To investigate this, we purified flag-tagged PARI to near homogeneity from insect cells, using a baculovirus expression system (Figure 5A), and performed ATP hydrolysis studies, using radiolabeled ATP. Unlike the bacterial RecA positive control, which contains a functional ATPase domain, PARI exhibited very weak ATP hydrolysis (Figure 5B, Supplemental Figure S5A). Together with the absence of detectable Walker motifs, this result argues that PARI is not an active helicase.

Next, we investigated whether recombinant PARI can promote the removal of RAD51 from single stranded DNA (Figure 5C). Preformed RAD51-ssDNA filaments were incubated with increasing concentrations of PARI, before the protein-DNA complexes were evaluated by native agarose gel electrophoresis. At sub-stoichiometric concentrations of PARI relative to RAD51, PARI had no significant effect on the stability of the single stranded RAD51-DNA filament. In contrast, when present at stoichimetric concentration relative to RAD51, PARI was able to promote the disassembly of the RAD51-DNA filament (Figure 5D, E). Demonstrating its specificity towards RAD51, PARI did not interact with bacterial RecA (Supplemental Figure S5B) and was unable to disrupt RecA filaments (Supplemental Figure S5C, D). Moreover, PARI failed to promote the disassembly of D-loop structures (data not shown), which contrasts with the potent D-loop disruption activity previously reported for another anti-recombinase, RTEL1 (Barber et al., 2008).

To examine a possible link between PARI and ATP hydrolysis of RAD51, we generated RAD51 nucleofilaments in the presence of the non-hydrolyzable ATP analog AMP-PMP. We observed that PARI is unable to disrupt filaments formed in the presence of AMP-PMP (Figure 5F, G), arguing that ATP hydrolysis by RAD51 is required for RAD51 nucleofilament disassembly by PARI. These results suggest that PARI acts stoichiometrically to remove RAD51 from DNA and suppress HR.

PARI knockdown improves genomic stability of HR-deficient cancer cell lines

Since PARI is an inhibitor of HR in human cells, we next tested if downregulation of PARI might suppress genomic instability in cells with an underlying genetic defect in HR. We reasoned that in these cells, unlike the situation in wildtype cells, the increased recombination conferred by the loss of PARI might in fact promote DNA repair and protect cells from the accumulation of DNA mutations and chromosomal aberrations. We downregulated PARI by RNA interference in Fanconi Anemia subtype D1, VU423 fibroblast cells, which harbor a biallelic hypomorphic mutation in BRCA2 (Howlett et al., 2002). Upon treatment of these cells with MMC, we quantified chromosomal aberrations in mitotic spreads. Strikingly, we observed that PARI knockdown in VU423 cells resulted in a decreased number of chromosomal aberrations compared to control knockdown (Figure 6A and Supplemental Figure S6A). PARI depletion from VU423 cells was also associated with increased resistance to the PARP-1 inhibitor AZD2281 (Figure 6B). PARP-1 inhibitor sensitivity is an indirect measure of HR deficiency, since cells with inactivated Base Excision Repair (BER) via inhibition of PARP-1, become hyper-dependent on HR for cellular viability (Bryant et al., 2005; Farmer et al., 2005). As another measure of HR, we quantified RAD51 foci in these cells. The reduced BRCA2 activity in VU423 cells due to the hypomorph mutation, possibly coupled with the RAD52 backup pathway for loading RAD51 (Feng et al., 2011), resulted in reduced RAD51 foci formation. PARI depletion resulted in increased baseline and IR-induced RAD51 foci (Figure 6C). These results argue that, in the absence of PARI, HR repair in BRCA2-deficient cells is improved, resulting in a protective effect against DNA damage.

We next used siRNA to attenuate the expression of BRCA2 in a U2OS cell line with an integrated direct repeat recombination reporter mentioned above. We observed that PARI co-depletion could indeed improve HR in cells with reduced BRCA2 activity (Figure 6D). The situation is not unique to BRCA2-deficient cells: a similar protection against damage-induced chromosomal aberrations was observed after knocking down PARI in DF138 fibroblast cells (Supplemental Figure S6B, C), another HR-deficient cell line with biallelic mutations in the Fanconi Anemia FANCJ gene (Litman et al., 2005). Similar results were obtained when knocking down PARI in FANCD2-deficient PD20 cells (data not shown). Thus, PARI knockdown improves genomic stability in cells with HR gene mutations.

Discussion

PARI inhibits recombination in human cells

Our results demonstrate that C12orf48/PARI acts to suppress inappropriate homologous recombination in higher eukaryotes (Figure 2, 3; see model in Supplemental Figure S6D). PARI has a PCNA-interaction domain and a SUMO-binding motif (Figure 1A), suggesting that this protein might function analogously to yeast Srs2 in higher eukaryotes. Previously, other human proteins have been suggested to act as Srs2. Human FBH1 helicase contains an active UvrD helicase domain, and cells overexpressing exogenous FBH1 exhibit reduced HR (Fugger et al., 2009). However, loss of FBH1 in either chicken cells (Kohzaki et al., 2007) or human cells (Moldovan and D'Andrea, data not shown) does not result in increased direct repeat recombination. Thus, the extent to which FBH1 acts as an anti-recombinase *in vivo* is unclear. Moreover, FBH1 has an additional domain, an F-box with ubiquitin ligase activity, and it is expressed in some yeast species, such as *S. pombe*, that also express Srs2. This suggests that FBH1 might have functions other than Srs2, which are conserved from yeast to humans.

Another anti-recombinase, RTEL1 was shown to inhibit recombination in worm and human cells (Barber et al., 2008). While RTEL1 is a functional analog of Srs2, it is a member of a different protein family, namely the RAD3 helicase family. Unlike Srs2 and PARI, RTEL1 does not disassemble pre-synaptic nucleofilaments, but rather disassembles D-loop structures formed following strand invasion. Thus, although both PARI and RTEL1 are recombination inhibitors, they act on different HR substrates (Supplemental Figure S6D). This situation provides an additional level of recombination control, allowing cells to interfere with HR at different steps in the process. Mammalian cells likely possess several distinct anti-recombination factors. This could explain why the hyper-recombination phenotype of PARI-deficient cells is relatively mild, since other Srs2 analogs might compensate in its absence. The RECQL5 helicase has also been implicated in the negative regulation of HR. Like PARI, RECQL5 deficiency leads to increased HR, and the RECQ5 protein is able to dismantle RAD51 nucleoprotein filaments *in vitro* (Hu et al., 2007).

PARI was initially cloned as a DNA/RNA binding protein (Borsu et al., 2000). Recently, PARI was proposed to play a role on BER, acting as a PARP-1 activator (Piao et al., 2011). While we cannot formally exclude an involvement of PARI in BER, our data strongly suggest that its major DNA repair *in vivo* function is through homologous recombination. Using artificial BER substrates, we found that *PARI*−/− DT40 cell extracts have normal BER (data not shown). Nevertheless, by promoting BER while at the same time inhibiting HR, PARI might be in the unique position to dictate the choice of DNA repair at a particular lesion.

PARI mode of action

RAD51 nucleofilament formation is essential for HR initiation. In its ATP-bound form RAD51 has high affinity for DNA. When ADP-bound, DNA affinity is weak. To promote HR, BRCA2 inhibits ATP-hydrolysis by RAD51, stabilizing the DNA-bound form (Jensen et al., 2010; Petalcorin et al., 2006). Srs2 was proposed to inhibit HR in yeast by activating the ATP-hydrolysis of RAD51 (Antony et al., 2009), which results in the ADP-bound RAD51 form and favors its release from DNA. Srs2 binding to RAD51 may induce a conformational change that results in ATP hydrolysis by RAD51. In the next step, Srs2 may use its own ATP hydrolysis to move along DNA, to the next RAD51 molecule. In this way, Srs2 is able to efficiently dismantle HR intermediates in a catalytic manner.

PARI lacks detectable Walker motifs and associated ATPase activity (Supplemental Figure S5). Even though we cannot formally exclude the possibility that some contaminating

activity present in our PARI preparation is involved in the RAD51 displacement activity observed (since we failed to identify a RAD51 interaction-deficient mutant), our results suggest that PARI acts in a stochiometric, non-catalytic mode to remove RAD51 from DNA *in vitro*, in a process that requires ATP hydrolysis by RAD51 (Figure 5). It is possible that the motor activity is provided in trans by a PARI associated helicase, which remains to be identified.

Another helicase-independent RAD51 removal activity was previously described for the helicase HELQ. HELQ removes RAD51 from double-stranded DNA, which may permit D-loop extension by DNA polymerases. In contrast to Srs2 and RTEL1, HELQ function is pro-recombinogenic and contributes to efficient DSB repair (Moldovan et al., 2010; Ward et al., 2010). Even though HELQ possesses motor activity, it is not essential for RAD51 removal *in vitro*. Instead, HELQ can act stoichiometrically towards RAD51 (Ward et al., 2010), similar to the situation we describe here for PARI (Figure 5).

PCNA-dependent activation of PARI

Since PARI lacks a detectable motor activity, it must be present at stoichiometric amounts at its sites of action in order to efficiently inhibit HR. However, PARI is found at very low levels in cells (Figure 2A and data not shown). This suggests that a co-factor may be required to concentrate PARI to the location where it is required. This might represent a mechanism to keep its activity in check, since its HR repair inhibition activity, if unconstrained, could be very toxic to cells. We have initially identified PARI as a PCNA-interacting protein (Figure 1). Moreover, we showed that chicken DT40 cells bearing a PARI deletion are corrected by introduction of wild-type human PARI, but not by PCNA interaction-deficient PARI variants (Figure 4). PCNA may act as a co-factor that concentrates PARI to its site of action. Since the fundamental function of PCNA is during DNA replication, and since PCNA is an essential component of the replication machinery, our results suggest that PARI functions to inhibit recombination at replication forks, reminiscent of Srs2 recruitment to replication forks in yeast (Papouli et al., 2005; Pfander et al., 2005). Srs2, however, has both PCNA-dependent and PCNA-independent functions (Pfander et al., 2005); in contrast, our data suggest that PARI has exclusively PCNA-dependent functions (Figure 4; see model in Supplemental Figure S6E). In yeast, PCNA is SUMOylated at the replication fork; this modification is bound by Srs2 (Hoege et al., 2002; Papouli et al., 2005; Pfander et al., 2005). This regulated interaction provides specificity for HR control in S-phase. We show that PCNA is SUMOylated in human cells, and that PARI interacts stronger with PCNA-SUMO1 (Figure 1F, Supplemental Figure S1C–F). These data suggest that PARI is a S-phase specific Srs2 analog.

PARI drives genomic instability in DNA repair deficient cancer cells

Detailed knowledge of HR mechanisms and its regulation is essential for developing new strategies for cancer prevention and treatment. PARI is overexpressed in pancreatic (Piao et al., 2011), breast and ovarian cancers (see http://www.genecards.org/cgi-bin/carddisp.pl?gene=C12orf48&search=c12orf48). PARI overexpression might represent a mechanism to drive genomic instability, by suppressing HR, in cells without mutations in HR factors.

Under normal conditions, dysregulated hyper-recombination, resulting from loss of Srs2 (Pfander et al., 2005), loss of PARI (Figure 2, 3), or upregulation of RAD51 (Martin et al., 2007), is associated with genomic instability. In contrast, increasing recombination in DNA repair deficient cells might result in protective effects (Schild and Wiese, 2010). In yeast, Srs2 deletion can suppress the DSB repair phenotype of HR deficient mutants (Fung et al., 2009). Depletion of PARI similarly suppressed the genomic instability of HR-deficient

VU423 (BRCA2 mutated) and DF138 (FANCJ mutated) cancer cells (Figure 6, Supplemental Figure S6). These results suggest that inhibition of PARI represents a potential chemoprotective therapy for patients with predisposition to cancer (like individuals harboring BRCA germline mutations). In these patients, HR deficiency drives genomic instability and accumulation of mutations that ultimately disrupt cell cycle control pathways, leading to cancer. Inactivation of PARI should increase HR in these cells, thus preventing genomic instability and protecting against cancer onset.

Experimental procedures

Materials and Methods

Cell culture and protein techniques—Human HeLa, 293T, U2OS, VU423 and DF138 cells were grown in DMEM (Invitrogen) supplemented with 15% Fetal Calf Serum. Denatured whole cell extracts were prepared by boiling cells in 100mM Tris, 4% SDS, 0.5M β-mercaptoethanol. To obtain native whole cell extracts for co-immunoprecipitation and GST-pulldown studies, cell were lysed in 50mM Tris pH 7, 1% NP40, 150mM NaCl containing protease inhibitors. For co-immunoprecipitation experiments lysates were prepared in the presence of the reversible crosslinking agents DSP (Thermo Scientific) −20mM final concentration. M2 Flag-agarose (Invitrogen) and Myc-agarose (Sigma) were used for immunoprecipitation. For cellular fractionation, cells were lysed in 50mM Tris pH 7, 0.1% NP40, 150mM NaCl. The chromatin fraction was separated from the soluble material by centrifugation, and washed repeatedly with the lysis buffer. Antibodies used for western blotting include PCNA (Abcam), Rad51 (Novus), FANCD2, Myc, and FLAG M5 (all from Santa Cruz Biotechnology), and rabbit polyclonal anti-PARI (obtained in our lab, by immunizing with a recombinant PARI fragment spanning the C-terminal 200 aminoacids). Western blot images and autoradiographs were quantified using the ImageJ software. For RAD51 immunofluorescence, cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature, followed by extraction with 0.3% Triton X100 for 10 more minutes on ice. The incubation with the primary antibody (Anti-RAD51, Santa Cruz Biotechnology) was done at 37°C.

Plasmids and siRNA—PARI cDNA was cloned from HeLa cells mRNA using standard techniques. For transient transfection, PARI was cloned into pCMV-Myc (Clonetech). Plasmid transfections were perfomed using Lipofectamine LTX (Invitrogen), while Lipofectamine RNAiMAX (Invitrogen) was used for siRNA transfection. SiRNA oligos were purchased from Invitrogen and Qiagen (sequences provided in Supplemental Information, together with additional experimental procedures).

DT40 methods—Standard DT40 methods were used (Hochegger et al., 2006). RAD51 immunofluorescence was performed as described above, using the anti-human RAD51 antibody (Santa Cruz Biotechnology). Cell extracts were obtained by boiling cells in 100mM Tris, 4% SDS, 0.5M β-mercaptoethanol. Gene targeting, sister chromatid exchanges, drug sensitivity assays and gene conversion assays using the SCneo system are described in the Supplemental Information, or were previously described (Hochegger et al., 2006).

Biochemical assays—For *in vitro* experiments, PARI was cloned into pFastBac HT1 (Invitrogen) and purified from baculovirus-infected SF9 insect cells by Flag M2 affinity purification, using a protocol previously employed for helicase purifications (Yusufzai and Kadonaga, 2008). RAD51 was purified from bacteria, as described (Barber et al., 2008). RAD51 displacement assays and D-loop recombination assays were performed as previously described (Barber et al., 2008; Ward et al., 2010).

Highlights

- PARI is a UvrD domain protein that interacts with PCNA and SUMO
- PARI is required for genomic stability in human and chicken cells
- PARI restricts inappropriate recombination by controlling RAD51 on DNA
- PARI downregulation improves recombination in Fanconi Anemia pathway deficient cells

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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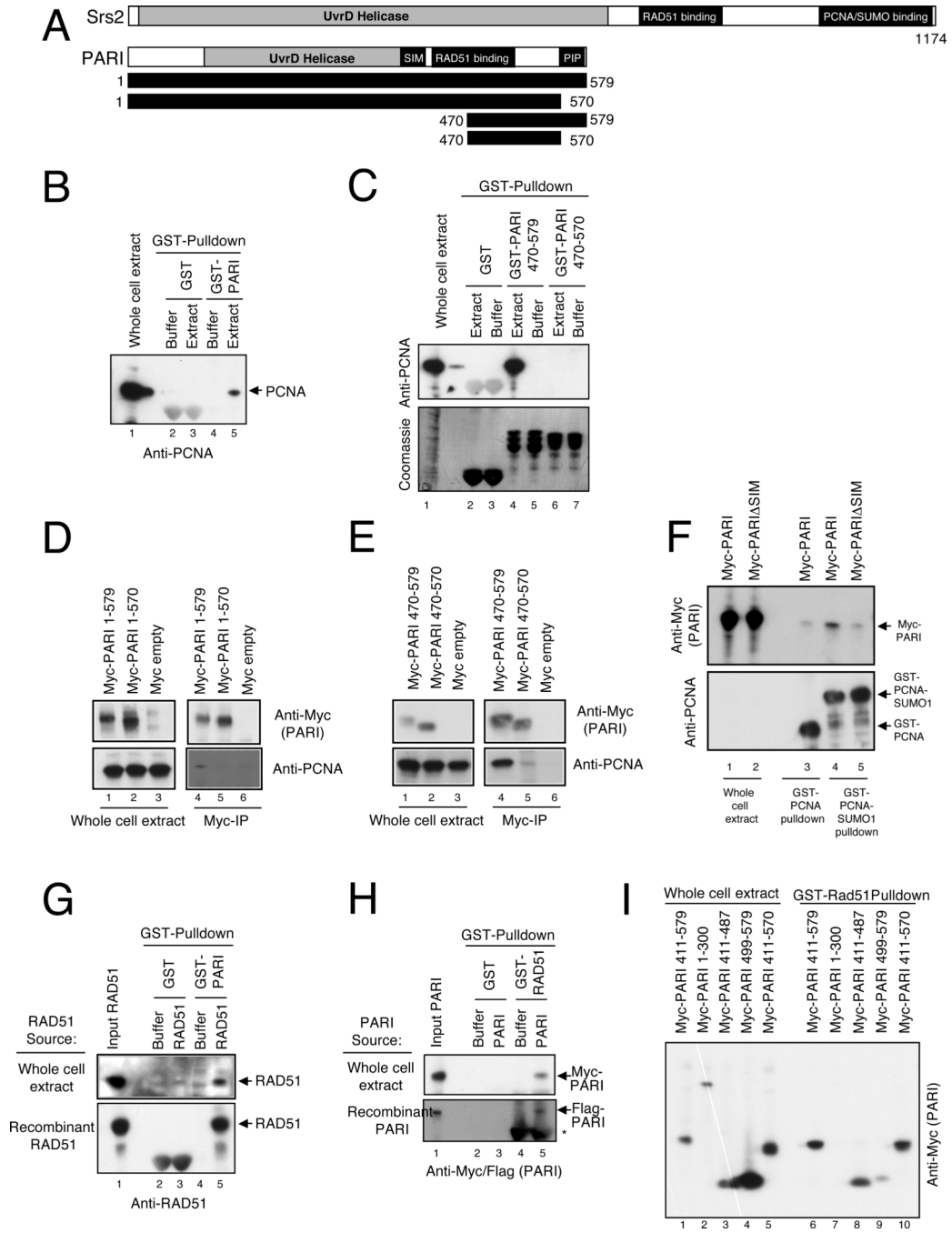

Figure 1. PARI is a PCNA-interacting protein

(A) Schematic representation of Srs2 and PARI domain structure (not to scale), and the PARI truncations used for interaction studies. The UvrD, SIM and PIP sequences are shown in Supplemental Figure S1A–C. (B) GST-Pulldown experiment showing that GST-tagged full-length PARI interacts with PCNA from whole cell extracts of HeLa cells. Recombinant GST-PARI is shown in Supplemental Figure S1D. (C) GST-Pulldowns using PARI truncations; the presence of the C-terminal PIP-box of PARI is required for PCNA interaction. (D) Anti-Myc immunoprecipitation from extracts of 293T cells expressing Myc-tagged PARI. Endogenous PCNA co-precipitates with full-length, but not PIP-box – deleted PARI. (E) Co-immunoprecipitation of PCNA with Myc-tagged PARI C-terminal constructs

required the presence of the PIP-box. (F) Wildtype and a PARI mutant in the hydrophobic patch of the SIM (356LLVL-PAAP, labeled PARIΔSIM), were expressed in 293T cells. Extracts were subjected to GST-Pulldowns with GST-PCNA and GST-PCNA-SUMO1 fusions (which are shown in Supplemental Figure S1D). A quantification of the binding strength is shown in Supplemental Figure S1J. (G–I) PARI interacts with RAD51. (G) GST-pulldowns showing that recombinant GST-PARI interacts with endogenous RAD51 from HeLa cell extracts (upper panel), as well as with recombinant RAD51 expressed and purified from bacteria (lower panel). (H) In a reciprocal experiment, GST-RAD51 (shown in Supplemental Figure S1D), was found to interact with Myc-tagged PARI expressed in HeLa cells (upper panel), and with recombinant Flag-tagged PARI purified from insect cells (lower panel). The asterisk denotes a crossreactive band (also in following panels). (I) RAD51 interacts with the region between aminoacids 411 and 487 of PARI. Myc-tagged PARI fragments were expressed in 293T cells and subjected to GST-RAD51 pulldowns.

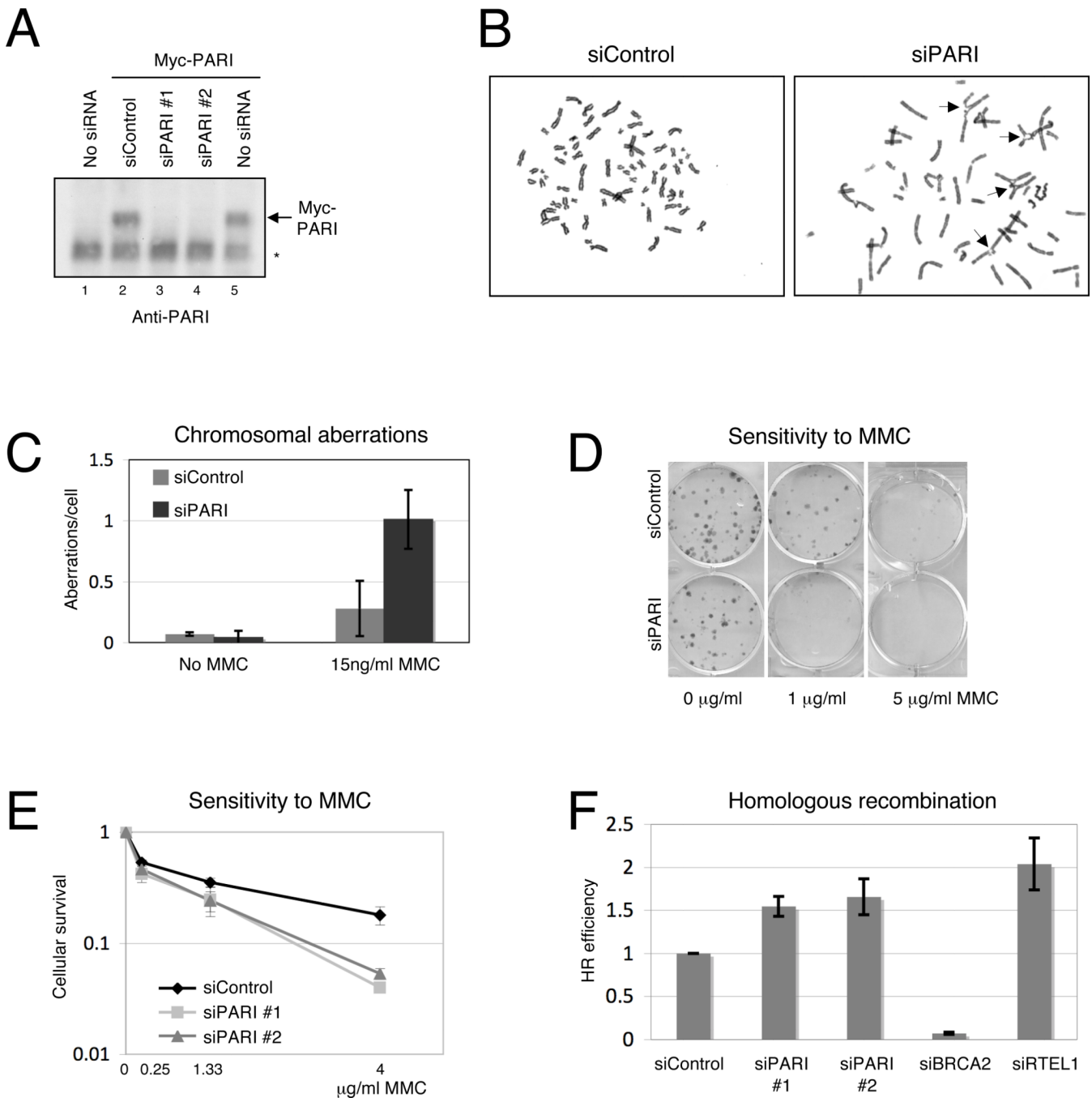

Figure 2. PARI is required for genomic stability and suppression of homologous recombination in human cells

(A) The siRNA sequences employed in this study efficiently downregulate the protein. An antibody raised against PARI can detect exogenously expressed PARI in Control, but not siRNA-treated cells. The antibody cannot detect endogenous PARI. Real-time PCR experiments confirmed the knockdown of endogenous PARI (Supplemental Figure S2A). (B) Metaphase spreads of MMC-treated 293T cells shows increased chromosomal aberrations following PARI knockdown. Representative aberrations are marked by arrows. (C) Quantification of chromosomal aberrations in Control and PARI-depleted cells. The average of two experiments is shown; at least 50 cells were counted in which experiments.

Error bars represent standard deviations. (D) Crystal violet staining showing that PARI-depleted HeLa cells are sensitive to MMC. (E) HeLa cells treated with siRNA targeting PARI show decreased cellular survival in the presence of MMC for three days. The average of two experiments is presented; error bars represent standard deviations. (F) Knockdown of PARI leads to increased homologous recombination, as measured using a direct repeat recombination assay (DR-GFP) in U2OS cells. Two to five independent experiments were averaged; standard deviations are shown as error bars.

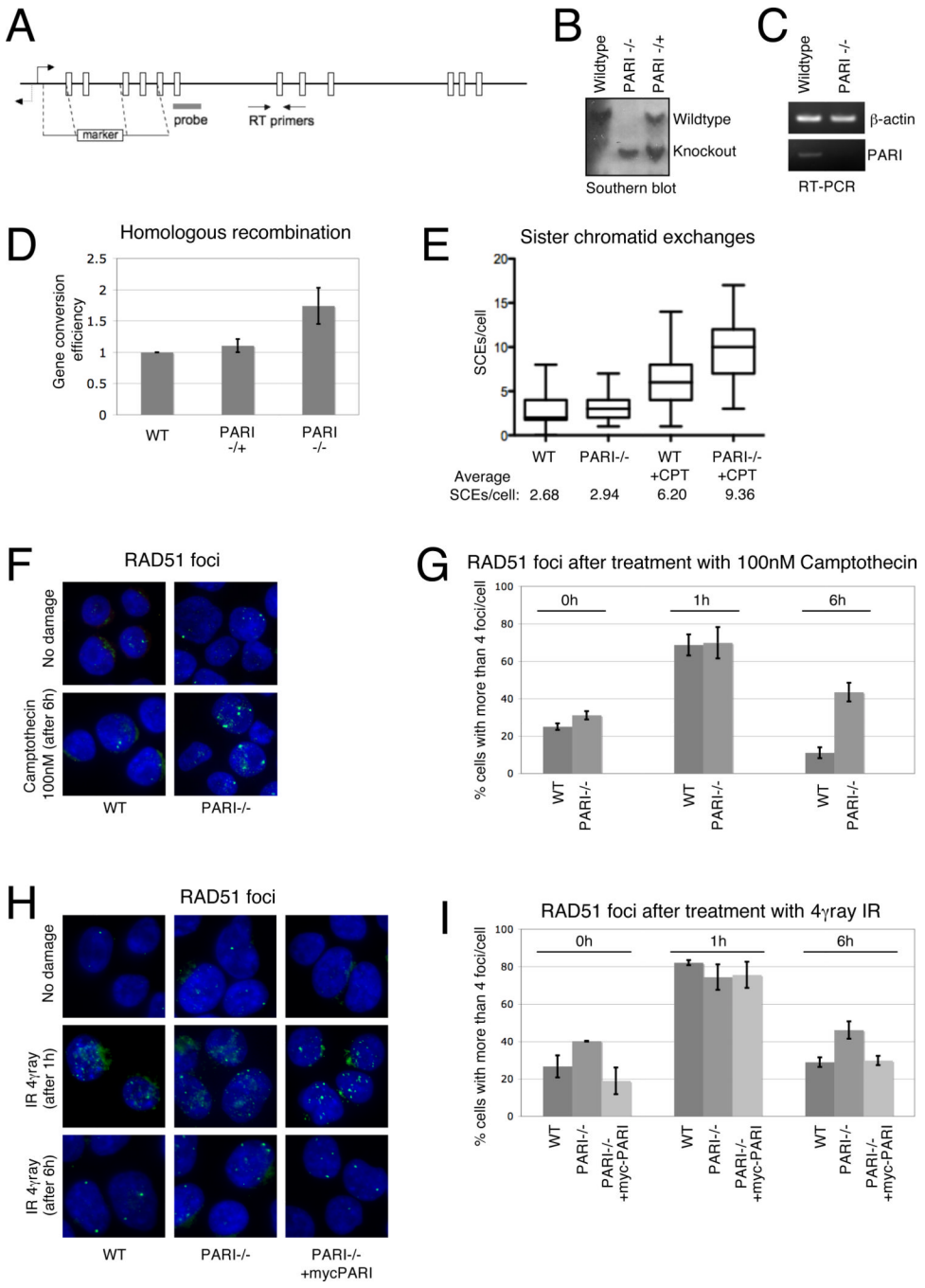

Figure 3. Hyper-recombination phenotype of chicken DT40 *PARI*−/− cells

(A) Graphic representation of the *PARI* gene locus on chicken chromosome 1. Also shown are insertion sites for the targeting cassette, as well as the positions for the Southern blot probe, and the RT-PCR primers used to detect the knockout alleles. (B) Southern blot analysis of the DT40 cell lines obtained, confirming the *PARI* knockout. (C) RT-PCR showing the loss of mRNA for PARI in the knockout cell line. (D) DT40-specific gene conversion assay (SCneo) showing that direct repeat recombination is increased in the absence of PARI. The average of at least two independent experiments is shown, with error bars representing standard deviations. (E) Sister chromatid exchanges in wildtype and *PARI* −/− DT40 cells. At least 50 cells were analyzed for each condition. Another representation

of this result is shown in Supplemental Figure 3A, B. (F) Representative micrograph showing increased Camptothecin-induced RAD51 foci in DT40 cells with PARI deletion. Data quantification is presented in panel (G). (G) Quantification of RAD51 foci in DT40 cells following treatment with 100nM Camptothecin. Cells were washed, released in normal media and analyzed at the time points indicated. Two experiments, with at least 40 to 125 cells analyzed for each situation, were averaged; error bars represent standard deviations. (H) RAD51 foci are increased in DT40 *PARI*−/− cells following IR exposure; introduction of human PARI corrects this phenotype. See quantification in panel (I). (I) Representation of RAD51 foci in DT40 cells treated with 4γray IR. Cells were analyzed at the indicated time points following radiation. The average of two experiments is shown. For each condition, 35 to 100 cells were analyzed. Standard deviations are presented as error bars.

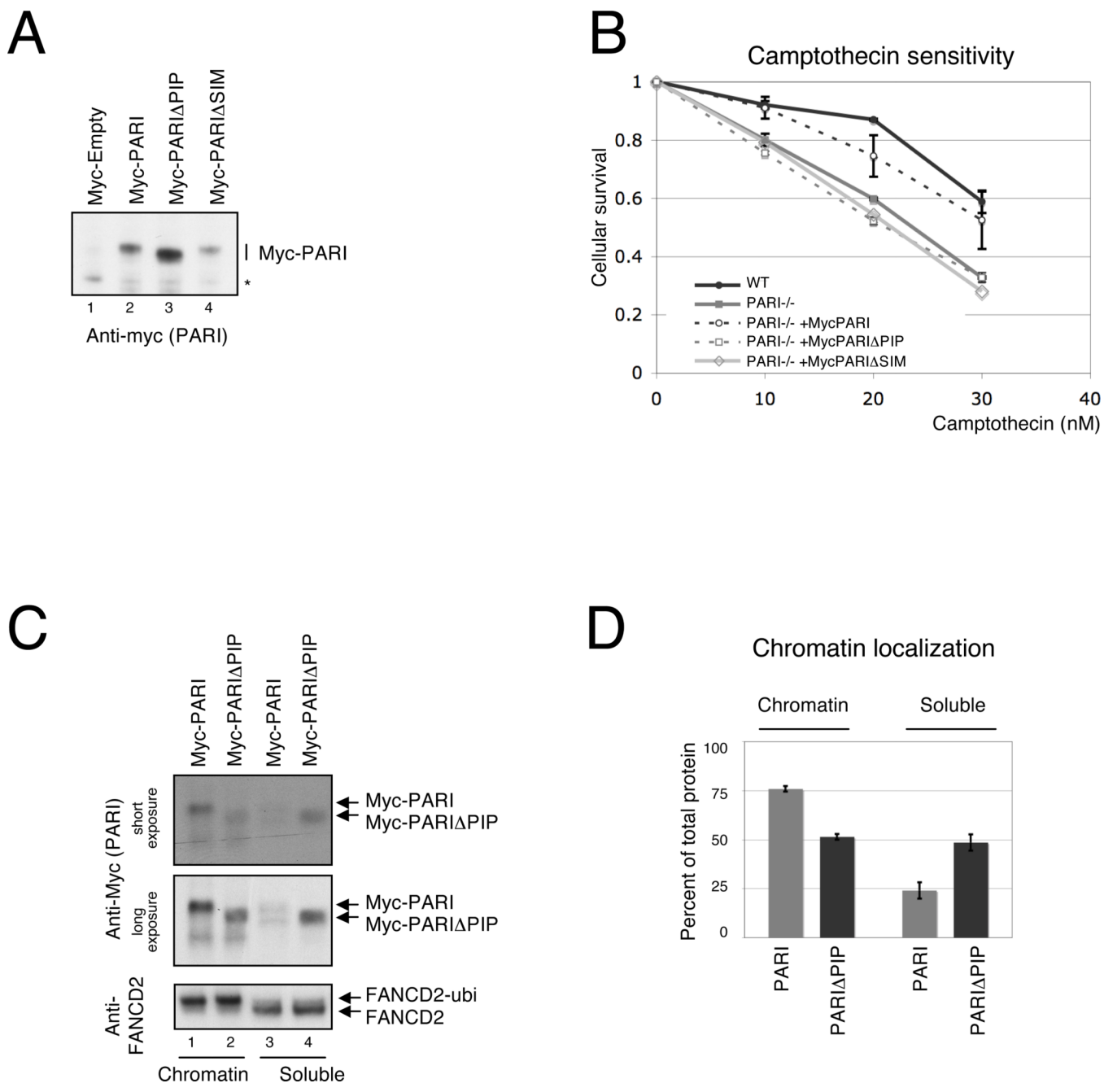

Figure 4. PARI functions require PCNA interaction

(A) Western blot showing the stable expression of human PARI, either full-length or lacking the C-terminal PIP-box, in chicken DT40 *PARI*−/− cells. (B) Wild-type, but not the PIP-box deletion (lacking aminoacids 571to 579) or the SIM mutant, can restore Camptothecin resistance in PARI-knocked-out DT40 cells. Cellular survival was measured after growth for four days in the presence of indicated Camptothecin amounts. Two independent experiments were averaged; error bars represent standard errors. (C, D) Fractionation of extracts of HeLa cells transfected with wild-type or PIP-box deleted, Myc-tagged PARI, and treated with 1mM hydroxyurea for 12h. The PCNA interaction-deficient PARI variant does not correctly localize to chromatin. (C) Anti-Myc western blotting was used to detect the localization of

exogenous PARI in fractionated extracts. As control for fractionation, an anti-FANCD2 blot, showing the exclusive chromatin localization of the ubiquitinated form, is also presented (lower panel). Quantification of this result is shown in panel (D). Bars represent the percentage of PARI protein present in different fractions, as averaged from two experiments; error bars are standard deviations.

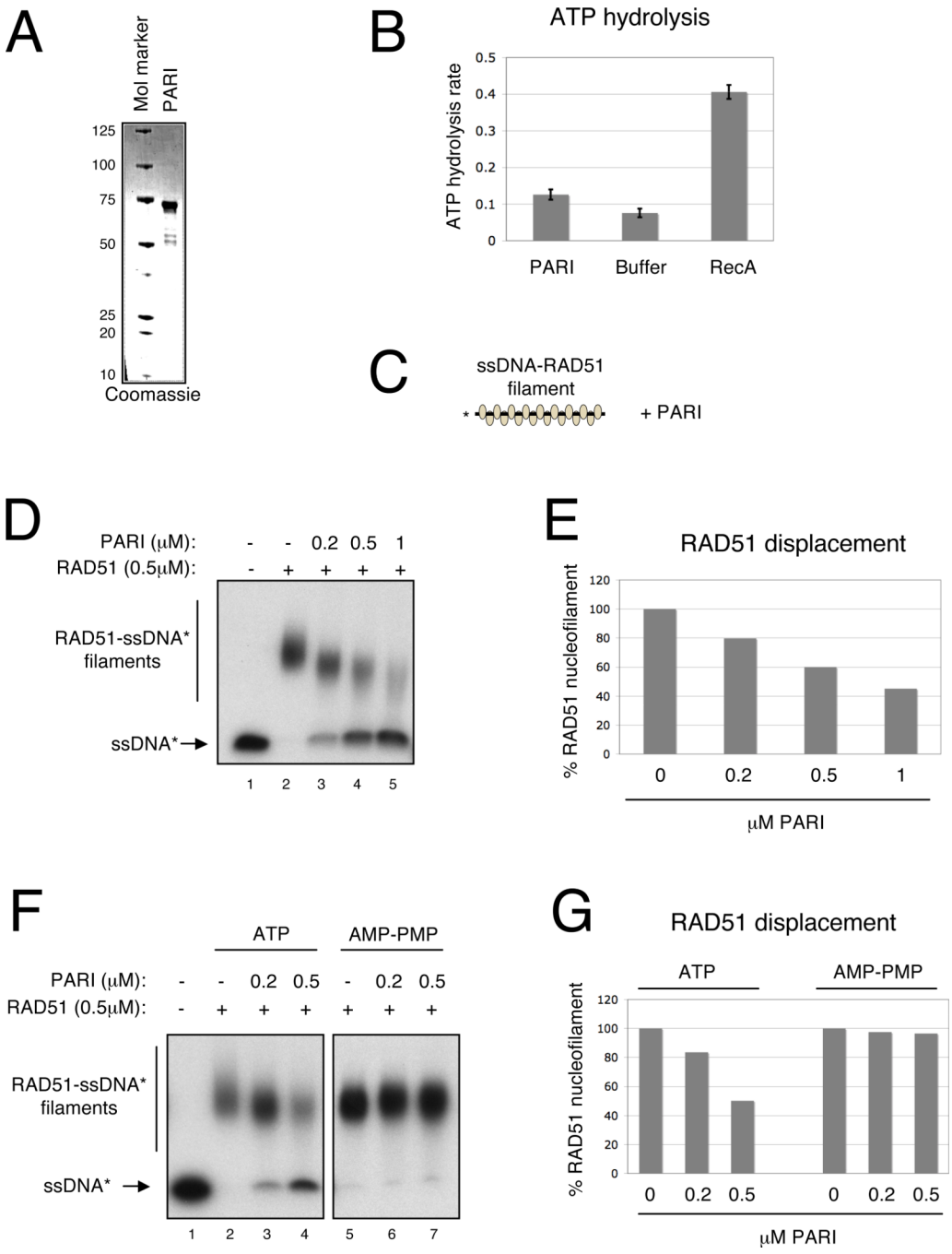

Figure 5. Disruption of RAD51 nucleofilaments by PARI *in vitro*

(A) The PARI preparation used for *in vitro* assays. The protein was purified from baculovirus-infected insect cells. (B) PARI has no ATPase activity. Shown is quantification of ATP hydrolysis by PARI and RecA as positive control, averaged from two independent experiments; error bars represent standard deviations. A representative experiment is shown is Supplemental Figure S5. (C) Schematic representation of the ssDNA-RAD51 filament disruption assay. (D, E) PARI removes RAD51 from ssDNA. (D) Autoradiograph of RAD51 nucleofilaments formed by incubation of recombinant RAD51 with radiolabeled ssDNA. Subsequent addition of PARI results in release of RAD51 from DNA. (E) Quantification of the ssDNA-RAD51 filament disruption result shown in the previous panel.

(F, G) ATP hydrolysis is required for RAD51 displacement by PARI. (F) Autoradiograph showing RAD51 ssDNA nucleofilaments formed in the presence of ATP or AMP-PMP. Addition of PARI only disrupted RAD51 filaments formed in the presence of ATP. (G) Quantification of the result shown in panel (F).

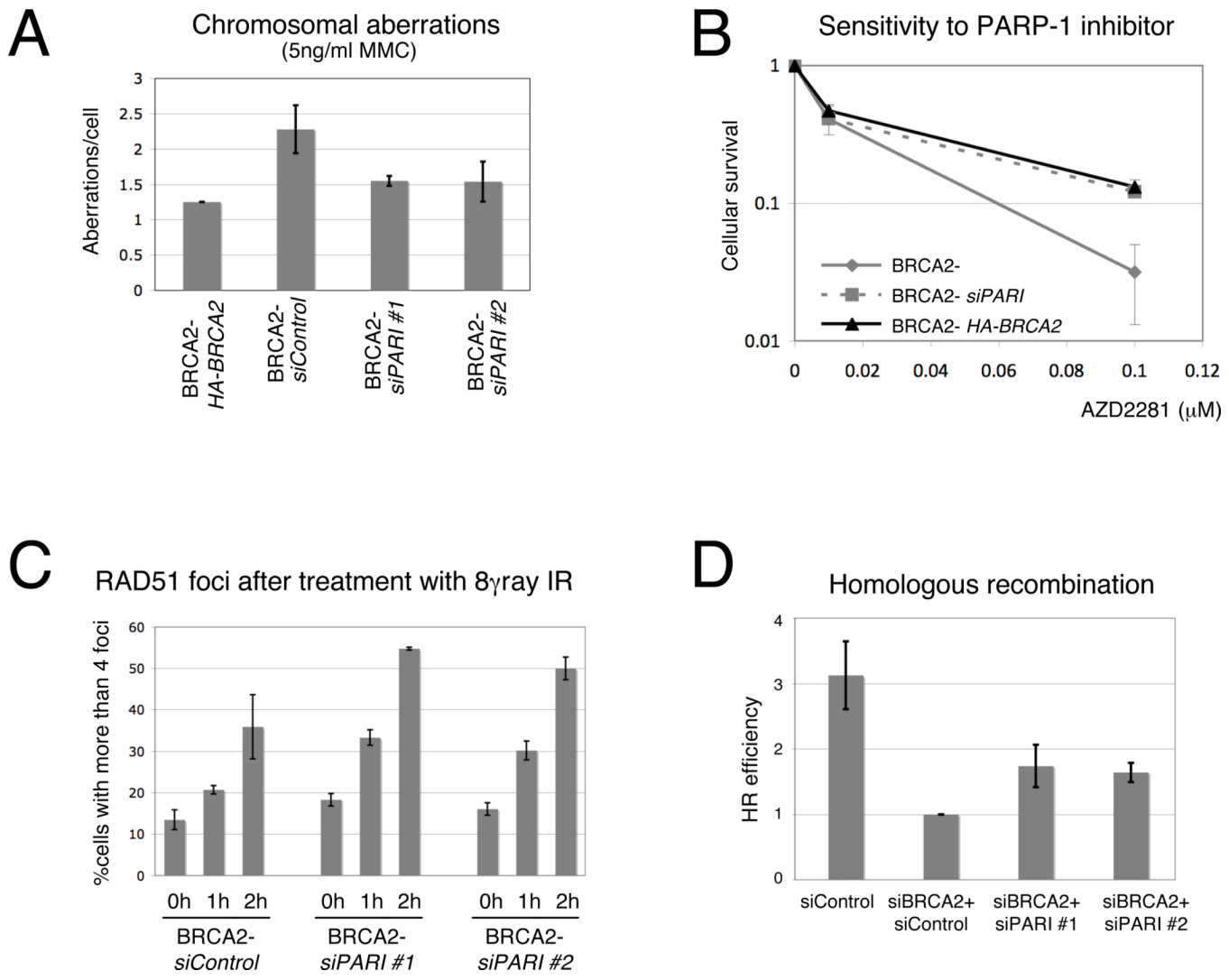

Figure 6. Improved genomic stability in BRCA2-deficient cells following PARI depletion

(A) Representation of chromosomal aberrations quantified from metaphase spreads of BRCA2-deficient VU423 cells (labeled BRCA2-). Cells were treated with Control or siRNA targeting PARI, and incubated for three days with 5ng/ml MMC. As control, VU423 cells in which BRCA2 cDNA was transfected were used. Two independent experiments were averaged; for each condition, at least 50 cells analyzed. Error bars represent standard deviations. The quantification of radial chromosomes is shown in Supplemental Figure S5A. (B) Sensitivity to PARP-1 inhibitor AZD2281 of VU423 cells is alleviated by PARI knockdown, or by BRCA2 re-introduction. The average of two experiments is presented; error bars represent standard deviations. (C) Baseline and IR-induced RAD51 foci are increased in VU423 cells following PARI depletion. Cells were analyzed at indicated time points after exposure to 8γray IR. At least 30 cells were analyzed for each condition. The average of two experiments is presented; error bars represent standard deviations. (D) Direct repeat recombination was measured in U2OS cells using the DR-GFP reporter. PARI knockdown partly alleviated the severe HR defect caused by BRCA2 depletion. The average of five independent experiments is presented. Error bars represent standard errors.