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Human RAD52 protein regulates homologous recombination and checkpoint function in BRCA2 deficient cells

Sukrit Mahajan[a], Komal Raina[a], Shalini Verma[a], B.J. Rao[b,*]

[a] Department of Biological Sciences, Tata Institute of Fundamental Research, Mumbai, India
[b] Indian Institute of Science Education and Research, Tirupati, India

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ABSTRACT

Cancer cells exhibit HR defects, increased proliferation and checkpoint aberrations. Tumour suppressor proteins, BRCA2 and p53 counteract such aberrant proliferation by checkpoint regulation. Intriguingly, chemo-resistant cancer cells, exhibiting mutated BRCA2 and p53 protein survive even with increased DNA damage accumulation. Such cancer cells show upregulation of RAD52 tumour suppressor protein implying that RAD52 might be providing survival advantage to cancer cells. To understand this paradoxical condition of a tumour suppressor protein facilitating cancer cell survival, in the current study, we investigate the role of RAD52 overexpression in BRCA2 deficient cells. We provide evidence that RAD52 protein alleviates HR inhibition imposed by p53 in BRCA2 deficient cells. In addition, we study the role of RAD52 protein during short replication stress in BRCA2 deficient cells. BRCA2 deficient cells exhibit excessive origin firing and checkpoint evasion in the presence of prevailing DNA damage. Interestingly, overexpression of RAD52 rescues the excessive origin firing and checkpoint defects observed in BRCA2 deficient cells, indicating RAD52 protein compensates for the loss of BRCA2 function. We show that RAD52 protein, just as BRCA2, interacts with pCHK1 checkpoint protein and helps maintain the checkpoint control in BRCA2 deficient cells during DNA damage response.

1. Introduction

Two different mechanisms of Rad51-dependent pathways have been proposed: Rad52 mechanism as found in yeast cells and the BRCA2 mechanism found in vertebrates (Wray et al., 2008). BRCA2 protein is known to recruit Rad51 to the repair sites on chromatin for mediating homology search and DNA strand invasion steps of homologous recombination (HR). BRCA2 protein deficiency is accompanied with sensitivity to cross-linking agents, defects in homologous recombination and DNA replication (Thorslund and West, 2007). Another HR player, Rad52 is a co-mediator of Rad51 function in homologous recombination and Rad52 mutants in yeast are highly sensitive to DNA damage (Hanamshet et al., 2016; Mortensen et al., 1996). Although RAD52 has been shown to stimulate Rad51 functions in vitro, RAD52 null mice and mammalian cells show no obvious phenotype (Mortensen et al., 1996; Rijkers et al., 1998). Therefore, the importance of RAD52 in mammalian cells remained elusive, until its inactivation was found to cause synthetic lethality in BRCA2 deficient cells and its compensatory role described during homologous recombination in a BRCA2 deficient background (Feng et al., 2011; Guo et al., 2011). RAD52 mediates Rad51 functions not only in the BRCA2 deficient cells but also in the cells deficient in PALB2 and BRCA1, two important proteins that help recruit BRCA2 to DSB repair foci (Lok et al., 2013). Unlike BRCA2 that stimulates HR, p53, another tumour suppressor protein, negatively regulates HR via direct interaction with various HR proteins (Linke et al., 2003; Verma and Rao, 2013; Janz and Wiesmüller, 2002). Consistent with this model, cells deficient in p53 display elevated levels of spontaneous and induced HR (Saintigny and Lopez, 2002). p53 mediated negative regulation of HR is countered by BRCA2 protein by its direct interaction with p53 protein (Verma and Rao, 2013; Marmorstein et al., 1998). Interestingly, tumours that show resistance to chemotherapy seem to exhibit upregulation of RAD52 and other HR components (Liang et al., 2013; Shi et al., 2012; Jewell et al., 2010) implying that an alternate repair pathway might be conferring survival advantage in such cells. All the studies conducted so far have involved knocking down RAD52 in the background of BRCA2 deficient cells leading to synthetic lethality, where it was not feasible to address the mechanistic role of RAD52. In the present study, we investigate the mechanistic basis of how overexpression of RAD52 protein might regulate DNA repair (HR and replication stress recovery) functions in BRCA2 deficient cells.

While the role of RAD52 is well studied in HR, not much is known

* Corresponding author.
E-mail address: Bjrao@iisertirupati.ac.in (B.J. Rao).

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about its function in replication fork repair following stress. Genotoxic stress results in cell cycle arrest which is implemented by checkpoint kinases, namely: CHK1 and CHK2 where the former kinase majorly responds to short replication stress, whereas the latter is activated during chronic stress leading to DSB formation (Zhang and Hunter, 2014; Stracker et al., 2009). CHK1 activation occurs via phosphorylation by ATR kinase to trigger checkpoint arrest till the damage is repaired. Active CHK1 (pCHK1) targets Cdc25 A phosphatase, which on phosphorylation undergoes proteasomal degradation, resulting in reduction of Cdk2/Cyclin A complex activity, thereby conferring checkpoint arrest (Zhang and Hunter, 2014). In addition, pCHK1 also induces chromatin release of Cdc45 (directly involved in origin firing) leading to inhibition of origin firing (Liu et al., 2006). Both mechanisms render replication machinery to stall or slow down till the damaged/stalled fork is repaired (Zhang and Hunter, 2014). In addition to phosphorylation based control by ATR, CHK1 activity is regulated via dephosphorylation by different phosphatases (Lu et al., 2005; Leung-Pineda et al., 2006). Moreover, the stable maintenance of phosphorylation mark on CHK1 also requires several modulatory proteins (Yarden et al., 2002, 2012; Chini et al., 2006; Wang et al., 2006), where the net-balance between all such counter-regulations results in checkpoint strength regulation during the cell cycle. Even though it is known that BRCA1 protein (upstream to the BRCA2-Rad51 axis) along with Claspin protein, interacts with CHK1 protein, thereby facilitating CHK1 activation and maintaining its phosphorylation status (Chini et al., 2006; Draga et al., 2015), however the details of the underlying mechanism are still unclear. It is surmised that phosphorylation of pCHK1 leads to structural change in the protein that sterically unmasks the kinase active site in the enzyme (Han et al., 2016) whose continued maintenance might require the pCHK1 stabilising proteins described above.

Interestingly, CHK1 protein also interacts with BRCA2 and Rad51 proteins, directly phosphorylating them for the formation of active Rad51 nucleofilament, especially during replication blockage (Bahassi et al., 2008). This study along with that of Yarden et al (Yarden et al., 2002) motivated us to investigate the status of pCHK1 axis in the context of BRCA2 protein, one of the players downstream to BRCA1. In this study, we probed for pCHK1 status in BRCA2 deficient cells during short replication stress and show that BRCA2 deficiency leads to a significant loss in pCHK1 level transiently and the cells lose the ability to undergo checkpoint arrest. Interestingly, RAD52 overexpression in BRCA2 deficient cells leads to restoration of checkpoint arrest during replication stress and the mitigation of excess origin firing observed in BRCA2 deficient cells, thereby phenocopying BRCA2 positive cells. Lastly, our demonstration of human RAD52 interacting with pCHK1 protein in mammalian cells for the first time, helps us propose a model where RAD52 protein molecularly compensates for the loss of BRCA2 functions in the cells.

2. Results

2.1. Human RAD52 interaction with p53 alleviates p53-mediated HR inhibition in BRCA2 deficient cells

In order to probe the mechanistic basis of how RAD52 protein expression impacts HR as well as replication stress in BRCA2 deficient cells, and whether the same involve undoing negative regulation of p53 on BRCA2 functions, we first tested whether RAD52 directly interacts with p53 protein, in vitro. N-terminal GST tagged human p53 recombinant protein bound to Glutathione sepharose beads pulled down a known interaction partner, Rad51 (37 kDa, positive control), but not *Chlamydomonas reinhardtii* protein UVI31 + (14 kDa, used as a negative control). In the same conditions, we observed that RAD52 protein (47 kDa, test protein) was also pulled down by GST tagged p53 bound to beads, suggesting direct interaction between these two proteins (Fig. 1A). Furthermore, GST tagged p53 protein bound only to the full length and the C terminal deletion construct of RAD52 protein, thereby revealing that the N-terminal region which is missing in all other four deletion constructs is important for interaction with p53, while C-terminal sequence is not essential (Fig. 1B, S1A). None of the deletion constructs and full length RAD52 protein were pulled down by empty GS beads in the absence of p53-GST, non-specifically. In order to verify whether these two proteins interact in human cells, we overexpressed RAD52 protein in EUFA423 cells (BRCA2 deficient cells used in subsequent experiments, Fig. S1B) and probed its interaction with p53 using co-immunoprecipitation (IP) assay. We pulled down RAD52 protein along with p53 using anti-p53 antibody in the immunoprecipitation (IP) assay as detected by western analysis (Fig. 1C and D). In the same lysates, reverse IP involving anti-RAD52-antibody pulldown revealed the capture of not only RAD52, but also p53 protein, thereby confirming interaction between these two proteins in cell lysates (Fig. 1C and D).

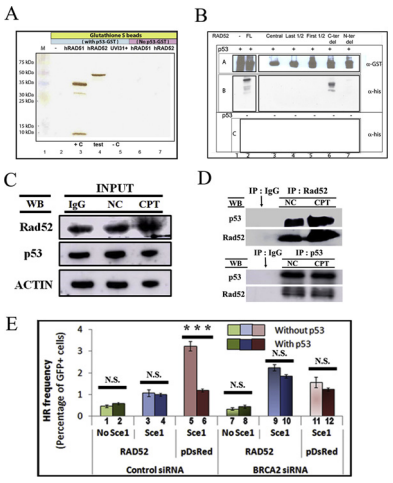

Fig. 1. p53 interacts with RAD52 both in vitro **and** in vivo.
(A) Equal amounts of p53 protein coated Glutathione-S beads were taken (lanes 2–5) to pull down the interactors from *E. coli* lysates overexpressing 6X-His tagged Rad51 (lane 3), RAD52 (lane 4) and UVI 31 + (lane 5) proteins. Anti-His western blot detects the presence of Rad51 and RAD52 as interactors of p53 that were pulled down. Rad51 and RAD52 were not bound by blank Glutathione-S beads in the absence of p53 (lanes 6 & 7).
(B) p53-GST mediated pull down of His-tagged RAD52 full length and deletion proteins in *E. coli* lysates. The pull down samples were analysed for the presence of RAD52 protein by anti-His western analysis (panel B). The western blot reveals the interaction of p53 with full length RAD52 (lane 2, Panel B) and its C-terminal deletion protein (lane 6, Panel B). p53 did not interact with other deletion constructs of RAD52 (Lanes 3,4,5 and 7, Panel B). Panel A is the anti-GST western showing equal amount of p53-GST on GS beads taken across the samples. Panel C is the anti-His blot of the samples in which RAD52 full length and deletion constructs were incubated with GS beads in the absence of p53 to check non-specific binding of RAD52 on the beads.
(C) Western blotting for Input samples of control IgG, untreated (NC) and cisplatin treated (CPT) samples for RAD52(DsRed tagged: 74 kDa), p53 and Actin. Input represents endogenous protein levels in the lysates used for IP.
(D) Co-IP with RAD52 and p53 antibody in vivo (i.e. in cells) in RAD52 stables. Anti-RAD52 antibody pull down samples were blotted with anti-p53-Ab and anti-RAD52-Ab. Similarly, in the reverse IP, anti-p53-Ab pull down was blotted with anti-p53-Ab and anti-RAD52-Ab. IgG lane represents a pull down with control IgG. NC: untreated; CPT: cisplatin treated samples.
(E). Quantitative analysis of HR frequency with GFP positive HEK293 cells expressed as percentage of total cells. The cells were transfected with *I-Sce1* (bars 3–6 and 9–12). The control sets with no *I-Sce1* transfection display spontaneous HR frequency (Lane 1–2 and 7–8). Different backgrounds of the cells in term of RAD52 overexpression and BRCA2 knockdown by siRNA are mentioned on the X-axis. pDsRed is the empty vector control without RAD52 gene. Control siRNA is a universal negative control that does not affect BRCA2 expression. The bars in the darker shade correspond to those with overexpression of p53. The cells were gated based on FSC and SSC values. For each sample, $n > 5 \times 10^5$ gated cells.

In order to study the relevance of p53-RAD52 interaction in homologous recombination (HR), we performed HR assay across two defective GFP cassettes sharing homology (Seluanov et al., 2010; Nakanishi et al., 2011) (Fig. S2A and B). Scoring for HR by FACS analysis, we observed that HR frequency was reduced in BRCA2 deficient cells compared to BRCA2 proficient cells (Fig. S1C and 1E, Compare Lanes 5 and 11). However, the overexpression of RAD52 in the cells with normal BRCA2 background suppressed HR perhaps due to some dominant-negative competition (Kim et al., 2001) by excess RAD52 protein (Fig. 1E; Compare lanes 3 and 5). Conversely, p53 overexpression in normal cells inhibited HR (Fig. 1E; Compare lanes 5 and 6), which is consistent with our earlier in vitro study where we showed that p53 interaction negates the stimulatory effect of BRCA2 on HR (Verma and Rao, 2013). Interestingly, RAD52 overexpressing BRCA2 deficient HEK293 cells showed no significant change in HR frequency between normal versus p53 overexpressed cells (Fig. 1E; Compare lanes 9 and 10), implying that RAD52 might be negating the inhibitory effect of p53 overexpression in these cells. In addition, overexpression of RAD52 showed stimulation of HR specifically in BRCA2 deficient cells (Fig. 1E; Compare Lanes 9 and 11). This result suggested the possibility of RAD52 overexpression compensating for the loss of BRCA2 function in the BRCA2 deficient cells, thereby mitigating the negative effect imposed by p53 protein, perhaps by interacting with it. We therefore surmise that RAD52 interaction with p53 might be physiologically relevant.

We noted that RAD52 overexpression led to a marginal increase in HR in BRCA2 deficient cells (generated by siRNA mediated inhibition) (compare 9 with 11 in Fig. 1E). We tested whether the overexpression effect improves further in cells where BRCA2 function is fully lost (EUFA423 cells are functionally BRCA2 null). Upon testing RAD52 overexpression stables in EUFA423 background, we observed a significant increase in the HR frequency, in both spontaneous and I-*Sce*1 induced HR, over that of control EUFA423 cells (Fig. S2C–D), suggesting that RAD52 over-expression compensates for the loss of BRCA2 function in HR repair. This result motivated us to test whether RAD52 overexpression possibly also compensates for the loss of other BRCA2 functions, namely, replication fork maintenance.

2.2. BRCA2 deficiency leads to increased new origin firing

Although BRCA2 role is well established during HR, its role during replication stress repair with respect to fork protection (Schlacher et al., 2011) and regulation of new origin firing have been described only more recently (Kim et al., 2014). In order to probe the role of RAD52 protein in replication fork maintenance function in BRCA2 functionally null cells (EUFA423), we first compared the replication fork dynamics of BRCA2 null cells (EUFA423) with that of its BRCA2 positive counterpart (EUFA423 + BRCA2 control cells) using short replication stress paradigm (2 mM HU treatment for 2 h) by performing DNA fibre assay (Schwab and Niedzwiedz, 2011; Dorn et al., 2009). We quantified fork restart, fork collapse and new origin firing during short replication stress (Fig. 2A and B) and observed no significant difference in fork restart or collapse frequency between these cell types, but uncovered higher frequency of new origin firing in BRCA2 null cells compared to positive controls, both in (-)HU (no replication stress) and (+)HU (replication stress) conditions (Fig. 2C and D), suggesting a possible dysregulation of replication origin control in cells devoid of BRCA2 function, a result consistent with reported findings of BRCA2 function during replication regulation (Kim et al., 2014). We note that HU treatment led to suppression of new origins with a concomitant rise in fork collapses in control BRCA2 positive cells compared to their (-) HU control conditions, a result consistent with replication stress response mounted by cells normally. However, in contrast, under the same conditions of HU treatment, BRCA2 null cells showed persistently higher level of new origins and lesser fork collapses (but statistically insignificant) compared to their BRCA2 positive counterparts, (Fig. 2C–E). On calculating fiber lengths, we found no significant difference in replication fork protection (CldU (Green) track length; Fig. S3A) in these two types of cells during control and replication stress conditions (Fig. S3B & C). Typically, reduced green track length (pre-stress replication fork length) following replication stress is interpreted as loss of fork protection, which was not observed in the current experiment. However new replication track length (IdU (Red) track) observed following HU treatment appeared to have been shorter in both types of cells (Fig. S3A–C), possibly suggesting slower progression of fork migration in cells during replication recovery. Interestingly, the observed track length difference between (-) HU versus (+) HU conditions of BRCA2 null cells was statistically non-significant while the same in BRCA2 positive control cells was significant (Fig. S3B & C), implying that BRCA2 null cells might have overcome the effect of reduced fork progression in post-HU recovery state. We surmised that this effect plus higher frequency of new origin firing might confer BRCA2 deficient cells faster rate of DNA replication (compared to BRCA2 positives) during recovery from replication stress. We tested this by scoring overall replication rate using an EdU (thymidine analogue) mediated labelling of new replication (following replication stress) as a function of time, which revealed that BRCA2 deficient cells (EUFA423) were distinctly faster in replication compared to BRCA2 positive control cells (EUFA423 + BRCA2) (Fig. 2F). Consequently, cells deficient in BRCA2 exhibited higher EdU positivity (compared to BRCA2 positives) even in (-) HU control conditions.

In conclusion, we show that BRCA2 deficiency results in enhanced origin firing leading to faster replication restart during replication stress recovery. In order to understand the mechanistic basis of replication fork dysregulation in BRCA2 null cells, we probed the checkpoint control in these cells.

2.3. BRCA2 deficiency leads to checkpoint evasion during replication stress

Since CHK1 kinase has been shown to regulate the S-phase progression by inhibiting origin firing in cells (Syljuåsen et al., 2005), we assayed for pCHK1 as a ratio of bulk CHK1 in the cells, during replication stress and recovery, as a function of time. We subjected the cells to replication stress treatment, following which they were allowed to recover in fresh medium for 2 and 4 h respectively and probed for pCHK1 and total CHK1 in whole cell extracts. As expected, we observed no significant difference between the pCHK1 levels of BRCA2 null (EUFA423) and BRCA2 positive (EUFA423 + BRCA2) cells in unperturbed control conditions. Interestingly, HU treated BRCA2 deficient cells showed a drastic fall in pCHK1 protein level as compared to BRCA2 positive cells (Fig. 3A and B). pCHK1 level in EUFA423 (BRCA2 null cells) were restored as a function of time by 4 h during recovery (from HU stress). In contrast, EUFA423 + BRCA2 (BRCA2 positive) cells showed no drop, but rather a marginal increase in expression of pCHK1 levels following HU treatment which got restored to untreated levels as a function of time, during recovery (Fig. 3A and B). In this experiment, among all the quantitative comparisons, we noted that only the attenuation of pCHK1 to CHK1 ratio in HU treated BRCA2 null cells was highly significant (compared to BRCA2 positive cells), thereby revealing that these cells exhibit a loss of checkpoint control during replication stress. The constitutive checkpoint strength (pCHK1/CHK1) diminishing in BRCA2 null cells following imposition of replication stress, is equated to checkpoint avoidance by the cells. In order to corroborate this effect, we monitored the cell cycle profile of these cells by FACS analysis. As expected of normal control cells, BRCA2 positive cells showed an increase in the percentage of S-phase cells with concomitant drop in G2 population, following HU treatment, compared to untreated controls, which came down to unstressed levels by 4 h of recovery (Fig. 3C and D). In contrast, in the same conditions, EUFA423 showed no such rise in S phase population nor concomitant fall in G2 population following HU treatment. In fact, the percentage of cells in S phase in BRCA2 deficient cells remained basal and low even after HU

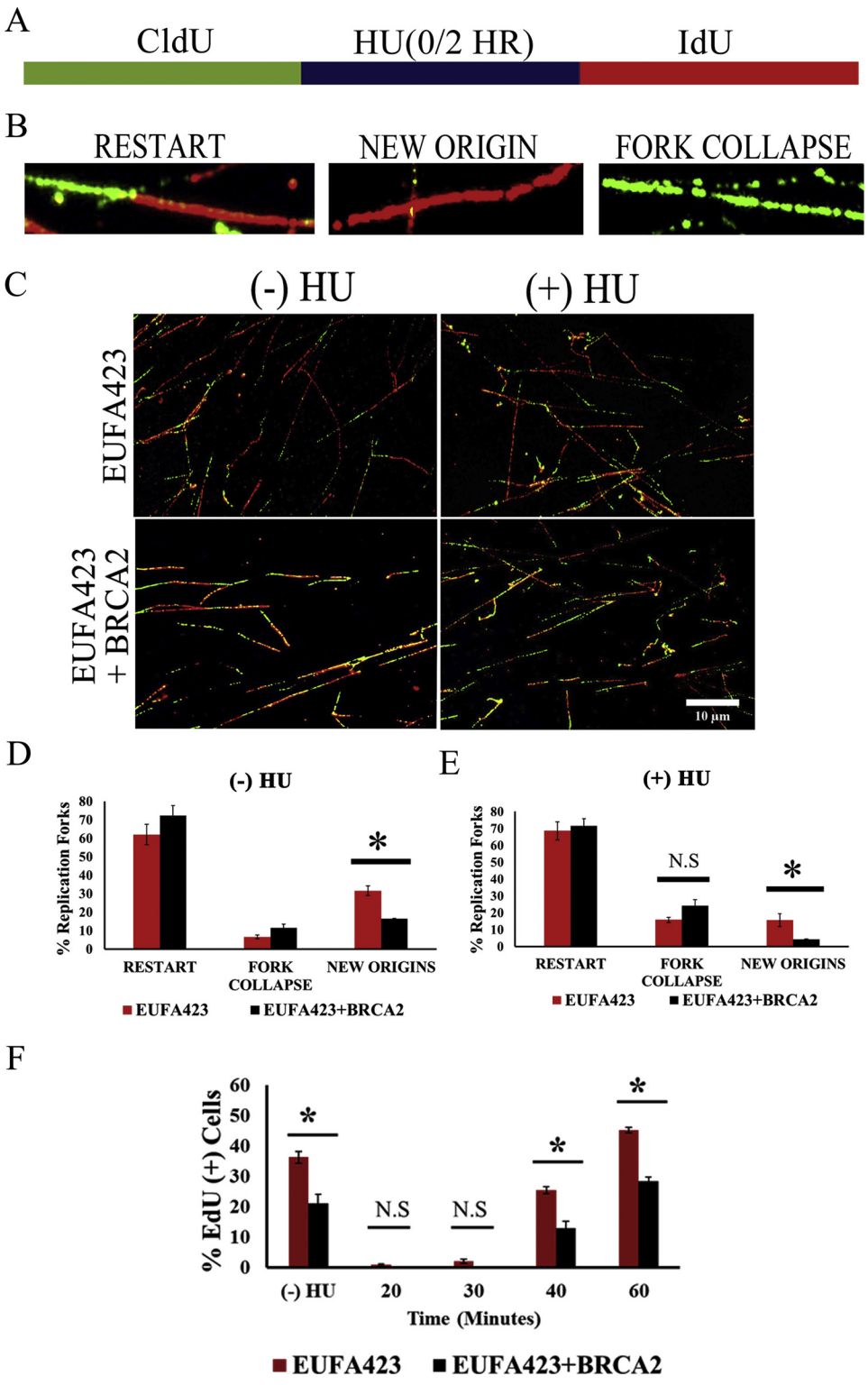

Fig. 2. Loss of BRCA2 function causes increased origin firing.
(A) Schematic: Cells were incubated in medium containing CldU for 20 min followed by 2 h of 2 mM HU. Cells were washed and incubated for 40 min in fresh medium containing IdU.
(B) Representative images of fibers depicting fork restart, new origin firing and fork collapse.
(C) DNA fiber populations of EUFA423 (BRCA2 deficient) and EUFA423 + BRCA2 (BRCA2 positive) cells in (-) HU and (+) HU conditions.
(D–E) Quantitative analysis of replication forks as a percentage of total fibres involving restart, fork collapse and new origin firing in (-) HU and (+) HU for EUFA423 and EUFA423 + BRCA2 cells. (n > 100; N = 3).
(F) Quantitative analysis of percentage EdU positive cells following HU stress removal. (N = 3, n = 100).

treatment and during the recovery stages (Fig. 3C and D), which suggested that BRCA2 deficient cells avoid checkpoint arrest after replication stress, a result consistent with the observed drop in pCHK1/CHK1 ratio in the previous experiment (Fig. 3A and B).

Having characterised the molecular and cellular phenotypes of BRCA2 deficient cells in control conditions as well as following replication stress, we wanted to probe whether expression of RAD52 affects the observed checkpoint avoidance and cell cycle defect in these cells. The mechanistic role of RAD52 in fork maintenance during replication stress has not been well explored, only changes associated with RAD52 protein following replication stress are described (Bhowmick et al., 2016; Sotiriou et al., 2016; Allen et al., 2011).

2.4. RAD52 overexpression rescues origin firing regulation during replication stress in BRCA2 deficient cells

As previously mentioned, Rad52 protein role has been studied extensively with respect to homologous recombination in yeast (Lee et al.,

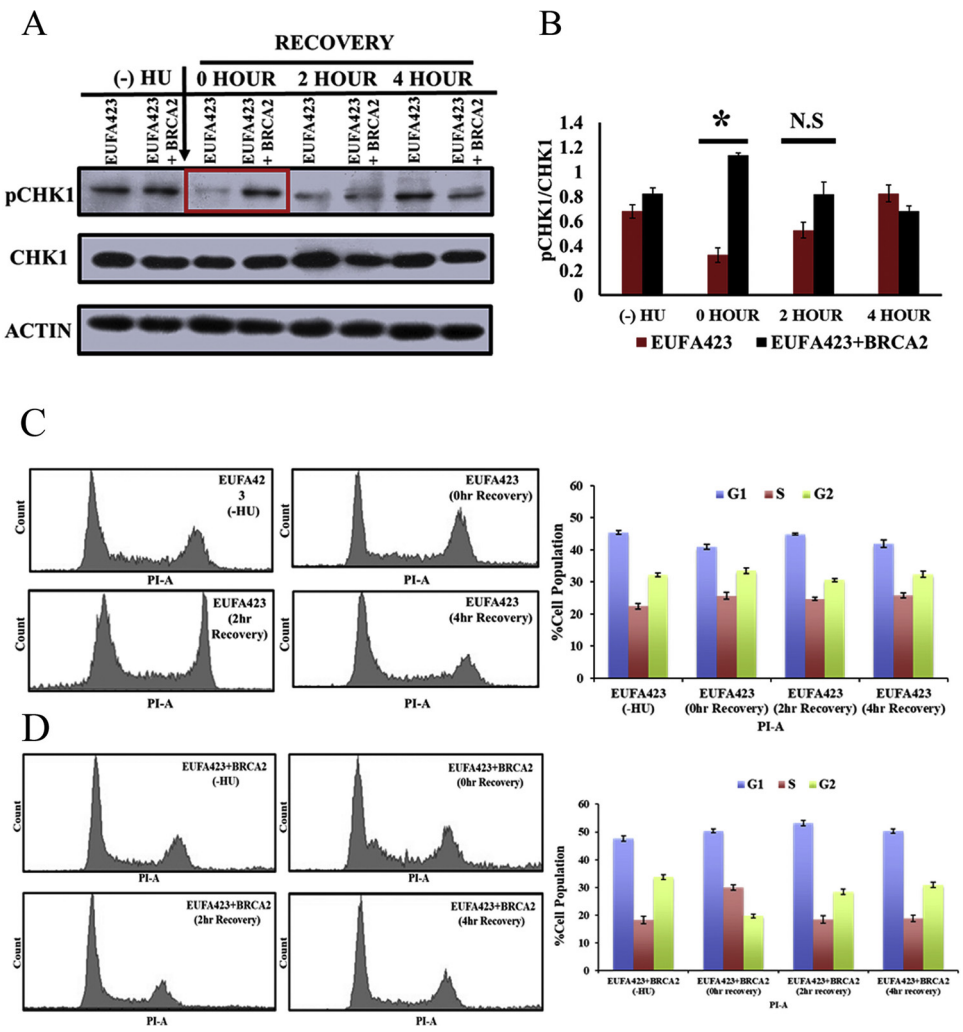

Fig. 3. Checkpoint defect observed in BRCA2 deficient cells.
(A) Whole cell lysates of EUFA423 and EUFA423 + BRCA2 were subjected to western blotting with pCHK1(56 kDa), CHK1(56 kDa) and Actin (42 kDa) antibodies for (-) HU, (+) HU, 2 h and 4 h recovery from HU. Stripping of the blot was performed to probe for proteins (pCHK1 and CHK1) at similar molecular weight.
(B) Quantitative analysis of band intensity of pCHK1/CHK1 in EUFA423 and EUFA423 + BRCA2. $P < 0.05$ ($N = 3$).
(C–D) Cell cycle profile of EUFA423 and EUFA423 + BRCA2 cells along with quantitative analysis depicting percentage of cells in G1, S and G2 phase. $P < 0.05$ ($N = 3$, $n = 6000$).

2003). It has been conjectured that RAD52 has lost its role in DSB repair in mammalian cells but retained its role in fork restart regulation post replication stress (Allen et al., 2011). There has been no experimental study so far that tested the role of RAD52 in replication fork maintenance during BRCA2 deficiency. Therefore, we wanted to probe the role of RAD52 protein during replication stress by comparing RAD52 overexpression stables with BRCA2 null cells (EUFA423). Cells overexpressing RAD52 protein showed new origin firing levels lower than that observed in BRCA2 null cells, both in normal as well as replication stress conditions (Fig. 4A–C), which suggested that RAD52 expression suppressed the enhanced firing of new origins observed in BRCA2 null cells. However, higher percentage of fork collapse was observed in RAD52 overexpressing BRCA2 nulls in both untreated and hydroxyurea treated conditions. These results suggest that RAD52 overexpression inhibited excessive origin firing in BRCA2 deficient cells; implying that RAD52 protein might compensate for the loss of BRCA2 function in these cells. Further, we analysed the replication track length to assess the fork stability, post-replication stress. We observed that as in BRCA2 deficient and proficient cells, RAD52 overexpressing cells also showed no change in fork stability in control versus replication stress conditions (CldU green track length) (Fig. S3D). However, newly replicated fork (IdU (Red) track length) appeared to be shorter following HU treatment in RAD52 overexpressing cells in comparison to (–) HU control, implying slower or delayed fork movement in these cells, a result similar to that of BRCA2 positive cells (Fig. S3C). In line with this, we observed that replication restart kinetics during recovery was slower in RAD52 overexpressing cells as compared to BRCA2 deficient cells (Fig. 4D), but similar to that of BRCA2 positive cells (Fig. 2F).

Interestingly, since the basal percentage of fork collapse was observed to be already high in RAD52 stables in comparison to BRCA2 null cells, it was important to assay for damage in these cells. We performed both alkaline and neutral comet assay, and found evidence of ssDNA and dsDNA breaks in RAD52 overexpressing cells even during control conditions. Comparison of untreated controls revealed that basal single and double strand breaks in RAD52 stables were higher than observed in BRCA2 null and BRCA2 positive cells (Fig. S4). On inducing stress by HU, both types of breaks further increased in RAD52 stables, and the increase was similar to that in BRCA2 null cells (Fig. S4), suggesting that over-expression of RAD52 might be rendering some level of dominant negative effect on the fork, thereby leading to enhanced level of fork collapse, as observed earlier (Fig. 4B and C). In contrast, BRCA2 positive cells showed only single strand breaks and were devoid of double strand DNA damages following HU treatment (Fig. S4).

All these results taken together suggested that RAD52 overexpression compensated for the absence of BRCA2 function in these cells. We probed the underlying mechanism further.

2.5. RAD52 protein restores checkpoint function in BRCA2 deficient cells

We uncovered that overexpressing RAD52 in BRCA2 deficient cells resulted in the restoration of reduced pCHK1 level observed in BRCA2 deficient cells following HU treatment (Fig. 5A and B), which “phenocopied” BRCA2 positive cells (Fig. 3A and B), suggesting that RAD52

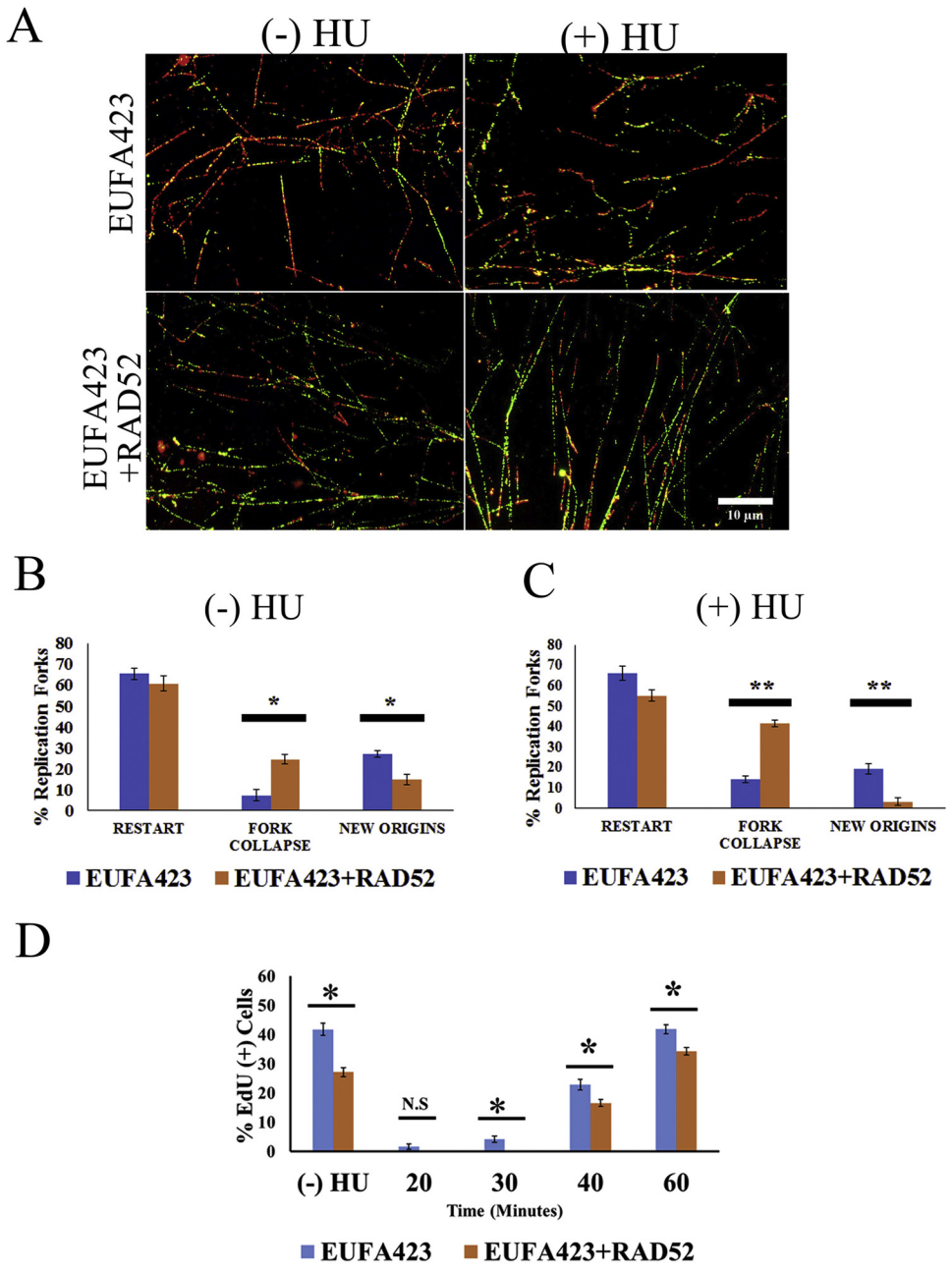

Fig. 4. RAD52 overexpression rescues enhanced origin firing in EUFA423.

(A) DNA fiber populations of EUFA423 and EUFA423 + RAD52 cells in (-) HU and (+) HU conditions. The conditions followed were as shown earlier in schematic (Fig. 2A).

(B–C) Quantitative analysis of replication forks as a percentage of total fibres involving restart, fork collapse and new origin firing in (-) HU and (+) HU conditions of EUFA423 and EUFA423 + RAD52 cells.

(D) Quantitative analysis of percentage EdU positive cells following hydroxyurea stress removal in EUFA423 and EUFA423 + RAD52 stables. (N = 3, n = 100).

protein might compensate for the loss of BRCA2 function in EUFA423 during replication stress. During recovery, the pCHK1 levels of both cell types (BRCA2 deficient and RAD52 overexpressing cells) reached similar values by about 4 h, which also compared well with that observed in no stress conditions. Further, on performing the cell cycle analysis, we observed that RAD52 stables regain S-phase arrest, as evidenced by higher S-phase fraction with concomitant drop in G2 (Fig. 5C and D), following HU-treatment which was in contrast with BRCA2 deficient cells that showed no S-phase arrest (Fig. 3E), a result further corroborating RAD52 mediated compensation for loss of BRCA2 function in cell cycle regulation during replication stress.

Restoration of pCHK1 level in BRCA2 nulls by the overexpression of RAD52 protein prompted us to assess whether RAD52 does indeed regulate CHK1/pCHK1 kinase by direct interaction. On performing co-immunoprecipitation assay with anti-RAD52 Ab, we pulled-down pCHK1 protein along with RAD52 protein, indicating direct interaction between these proteins. Reverse co-IP with pCHK1-Ab pulled-down RAD52 along with pCHK1, thus confirming the interaction (Fig. 5E–G). This interaction was evident even in HU untreated cells, which appears to persist following HU treatment. Based on these results, we conjecture that RAD52 protein performs checkpoint regulation function perhaps via a mechanism similar to that of BRCA2 involving pCHK1 axis.

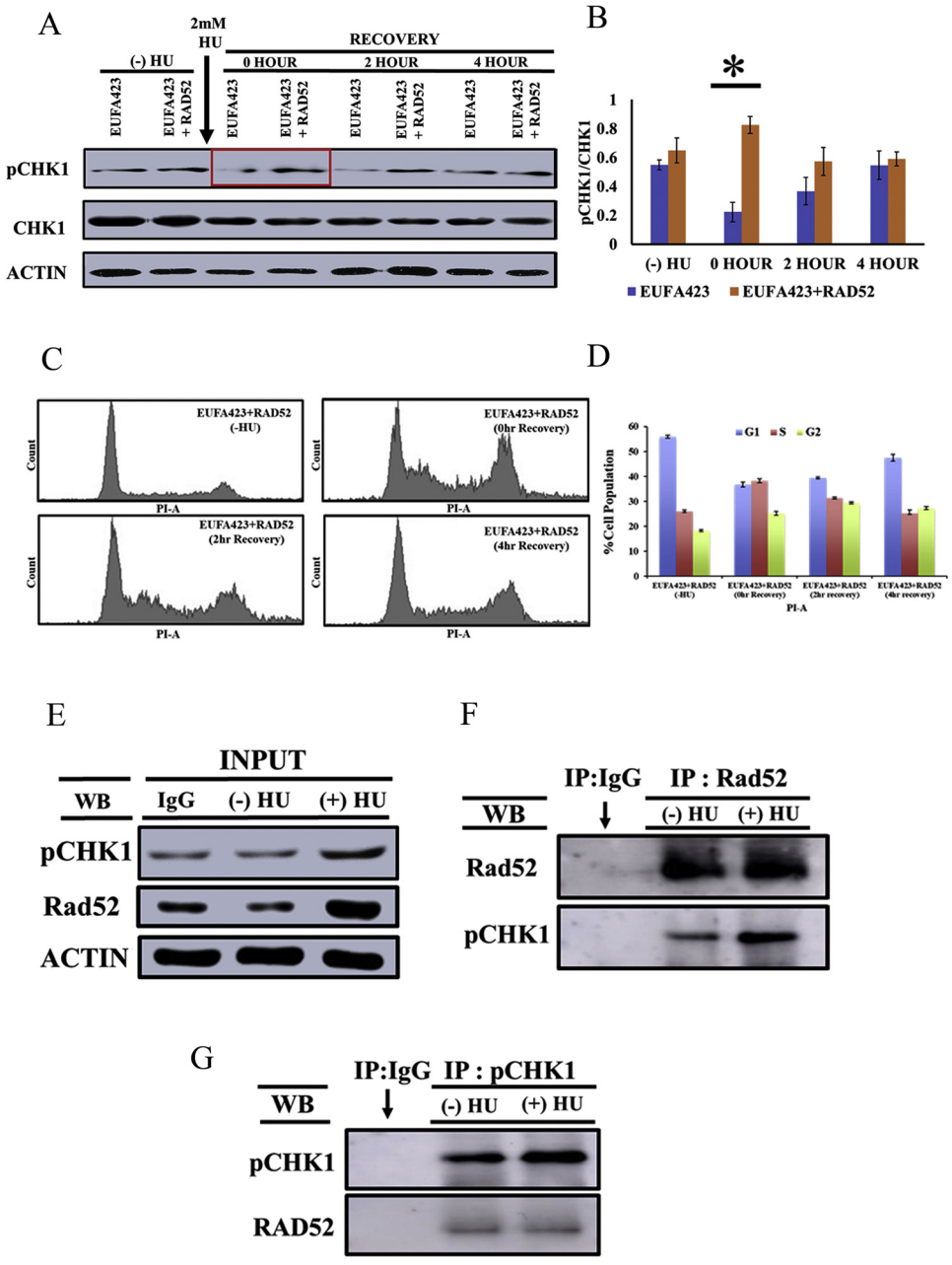

Fig. 5. RAD52 inhibits increased new origin firing by interacting with pCHK1.
(A) Whole cell lysates of EUFA423 and EUFA423 + RAD52 cells were subjected to western blotting with pCHK1(56 kDa), CHK1(56 kDa) and Actin (42 kDa) antibodies for (-) HU, (+) HU, 2 h and 4 h recovery from HU.
(B) Quantitative analysis of pCHK1/CHK1 band intensity for EUFA423 and EUFA423 + RAD52 cells. $P < 0.05$ (N = 3).
(C) Cell cycle profiling of EUFA423 + RAD52 cells in (-)HU and (+)HU conditions along with 2 h and 4 h of recovery. (N = 3, n = 10,000).
(D) Quantitative analysis of cell cycle profiling depicting percentage of cells in G1, S and G2 phase for EUFA423 + RAD52 cells.
(E) Endogenous levels of RAD52 (DsRed-tagged: 74 kDa), pCHK1 (56 kDa) in RAD52 stables and Actin (42 kDa) in IgG, (-)HU and (+)HU samples.
(F) Western blotting of RAD52 pull-down with antibody against pChk1 and RAD52(DsRed-tagged:74 kDa). IgG pull down performed using Rabbit Anti-IgG.
(G) Western blotting of pCHK1 pull-down with antibody against pChk1 and RAD52. IgG: control IgG. IgG pull down performed using Rabbit Anti-IgG.

2.6. ATR and ATM activity is unperturbed during stress conditions in BRCA2 deficient cells

CHK1 protein is the direct target of phosphorylation by ATR, which is actively involved in regulating the DNA damage repair dynamics during short replication stress. Since EUFA423 cells showed abrogation of the CHK1 checkpoint response during stress conditions, it was important to check for other markers of ATR activity to understand what might be happening upstream in the ATR signalling pathway. Therefore, we probed for phosphorylation status of γH2AX and pRPA32, direct targets of ATR during DDR response, in order to assay for DNA damage repair activation and attenuation in BRCA2 deficient cells. Using immunofluorescence assays, we probed for levels of γH2AX and pRPA32 in EUFA423, EUFA423 + BRCA2 and EUFA423 + RAD52 cells. BRCA2 deficient cells showed high baseline expression of γH2AX as compared to BRCA2 proficient cells and Rad52 stables (Fig.6A–C). The expression levels of γH2AX increased during stress conditions in EUFA423 cells, suggesting that the ATR-γH2AX axis is intact. The results when compared to BRCA2 positive cells and RAD52 stables, showed a linear correlation, with respect to upregulation of γH2AX during stress conditions. As expected, the recovery in BRCA2 proficient cells and RAD52 overexpressed cells was significantly faster in comparison to BRCA2 deficient cells, in which γH2AX levels persist. This was also reflected in the expression levels of RPA32, which showed upregulation during stress conditions in a BRCA2 deficient background.

We probed further to determine the activity of ATR (Fig. 6D–F) in these cells. During unstressed conditions, a baseline level of pATR expression was observed which increased significantly during the stress conditions in BRCA2 deficient cells, which was comparable to the expression levels in BRCA2 proficient cells and RAD52 stables. The expression recovers to un-stressed condition status by 4 h of recovery.

Subsequently, we also checked for the expression pattern of ATM (Fig. 6G–I) in these cells since we observed DSBs in the Comet Assays (Fig. S4). We observe a significantly high amount of expression of pATM in EUFA423 cells, in comparison to BRCA2 positive cells suggesting that ATM is actively involved in the DNA damage repair in

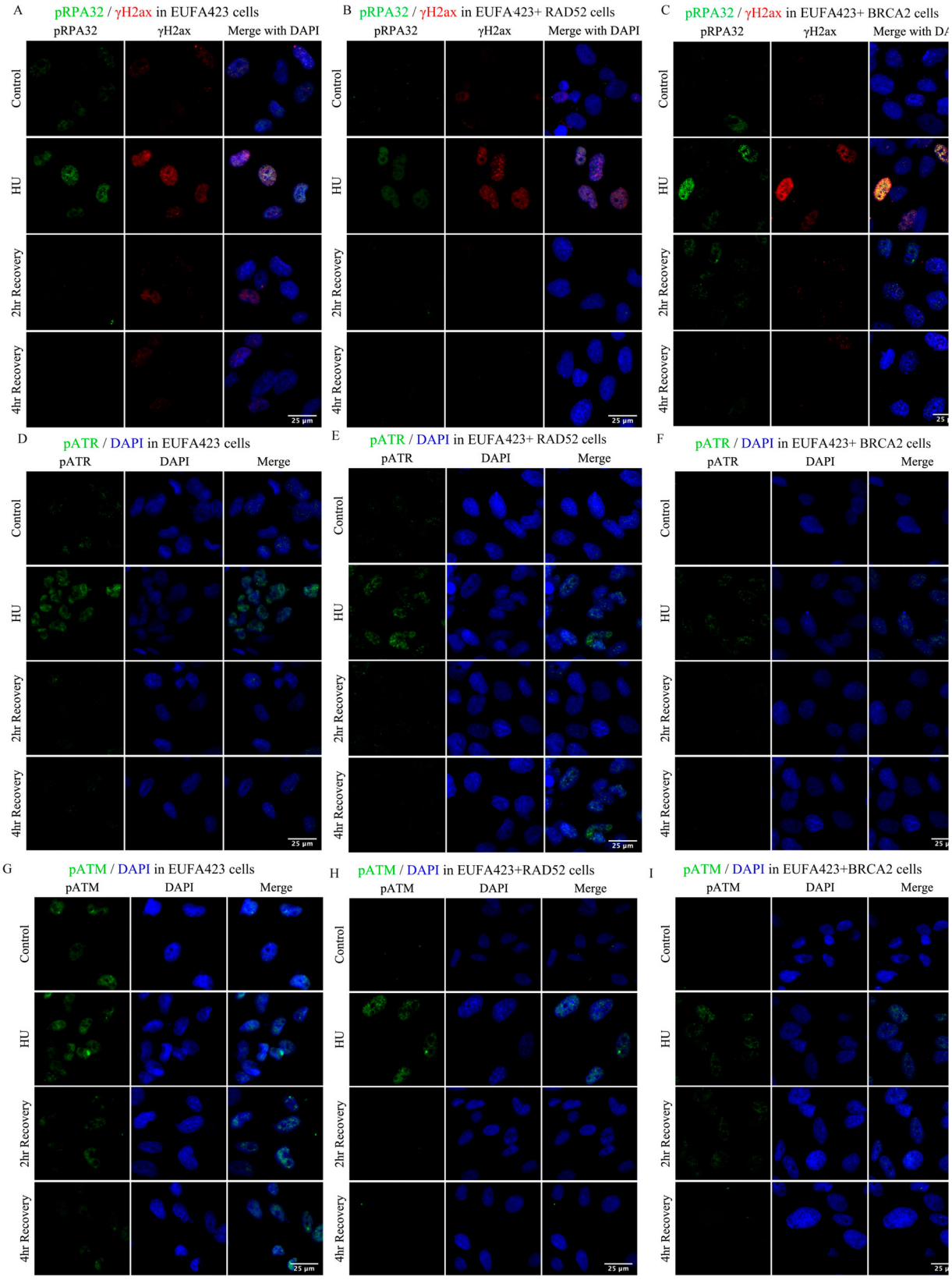

Fig. 6. ATR and ATM activity is unperturbed during stress conditions in BRCA2 deficient cells.
(A–C) pRPA32(green) and γH2AX (red) staining in EUFA423(Panel A), EUFA423 + BRCA2 (Panel B) and EUFA423 + RAD52 (Panel C) cells.
(D–F) pATR staining in EUFA423(Panel D), EUFA423 + BRCA2(Panel E) and EUFA423 + RAD52 (Panel F).
(G–I) ATM staining in EUFA423 (Panel G), EUFA423 + BRCA2 (Panel H) and EUFA423 + BRCA2 (Panel I).

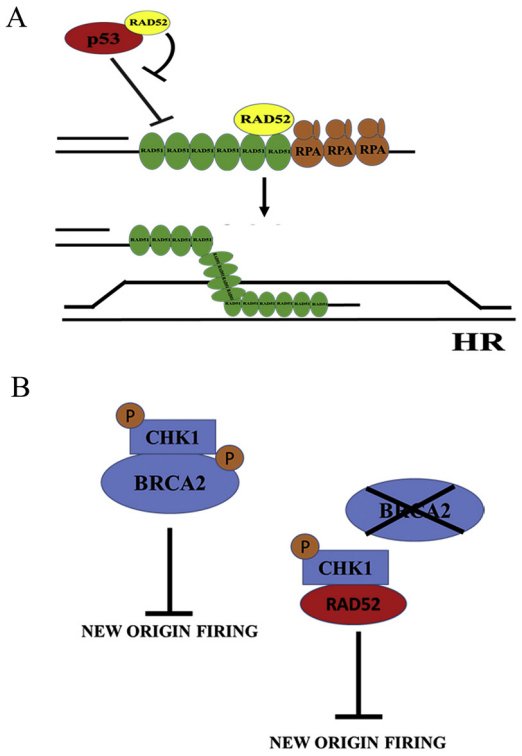

Fig. 7. Model.
(A) RAD52 protein suppresses the inhibitory effect of p53 on HR by interacting with p53.
(B) BRCA2 protein interacts with pCHK1 thereby maintaining its phosphorylation status. Similarly, RAD52 protein interacts with pCHK1 thereby maintaining its phosphorylation in the absence of BRCA2 protein in vivo.

BRCA2 deficient cells, along with ATR during HU induced stress. This result also supported our observations from the Comet Assay performed in order to determine the amount of DNA damage caused upon HU treatment. The DSBs observed in EUFA423 on HU treatment are repaired by ATM, which was found to be active in these cells, along with ATR. These results further strengthen our claim suggesting that the checkpoint evasion exhibited by BRCA2 deficient cells is not a result of perturbed ATR or ATM axes, but due to perturbation of the BRCA2-CHK1 axis, required for checkpoint regulation.

We discuss our results according to the model proposed in Fig. 7.

3. Discussion

Cancer cells deficient in BRCA2 protein, show increased proliferation, HR defects, and checkpoint aberrations, resulting in genomic instability. Since RAD52 protein has been shown to regulate HR in BRCA2 deficient cells (Feng et al., 2011), one would surmise that the protein mitigates DNA damages in the cells by repair and provides survival advantage to the cells. Also, melanomas relapsing post chemotherapy treatment exhibit overexpressed levels of RAD52 and other HR players (Jewell et al., 2010) suggesting that an alternate pathway involving RAD52 might be rescuing these cells from damage. While previously, RAD52 protein has been knocked down in BRCA2 deficient cells in order to show synthetic lethality between the two pathways (Feng et al., 2011), no RAD52 overexpression studies have been carried out in BRCA2 deficient cells so far.

In the current study, we attempt to understand whether indeed RAD52 might be providing survival advantage to the cells by regulating homologous recombination and replication stress (Mazouzi et al., 2014; Zeman and Cimprich, 2015) in the absence of BRCA2 protein function. Earlier it has been shown that RAD52 interacts with several repair proteins, such as RPA, Rad51 and PCNA (Linke et al., 2003; Mazouzi et al., 2014) here we uncover another important interactor of RAD52: we show its interaction with p53 in the context of HR repair. Our study provides definitive evidence that RAD52 expression not only compensates for the loss of BRCA2 protein function by rescuing HR but also negates the inhibitory effect of p53 on HR (Fig. 1), most likely via direct interaction with p53 protein (Fig. 6A).

It has been conjectured that during evolution, vertebrate RAD52 protein might have retained its role in replication fork repair while losing its function in HR to proteins such as BRCA2 (Allen et al., 2011), which prompted us to probe the role of RAD52 in the context of replication stress. In the current study, we focused on transient replication stress (2 mM HU treatment for 2 h) rather than DSB inducing chronic stress whose repair is HR dependent. We observed no significant difference in the fork restart frequencies of BRCA2 null and BRCA2 positive cells, implying that BRCA2 may not have any role in fork restart, which is consistent with the previous finding that XRCC3 protein mediates fork restart during recovery from short replication stress in these cells (Zeman and Cimprich, 2015). In contrast, BRCA2 nulls exhibiting excessive firing of origins during replication stress recovery indicates that BRCA2 might be directly involved in inhibiting new origins from firing. In fact, BRCA2 has been shown to interact with Cdc45 protein, which is part of the origin firing complex (Romanova et al., 2004). The exact nature of this interaction and the mechanism of origin firing inhibition by BRCA2 remains to be resolved.

BRCA2 protein coordinates cell cycle kinases such as Plk1 and CDK1 which in turn are known to phosphorylate BRCA2 protein itself (Petermann et al., 2010). Interestingly, an important cell cycle kinase, CHK1 has also been reported to phosphorylate BRCA2 protein at the C-terminal, facilitating BRCA2 assisted Rad51 nucleofilament formation (Bahassi et al., 2008). In line with this study, the proposal made by Yarden et al., 2012 where BRCA1 interacts with CHK1 kinase, which in turn facilitates the maintenance of pCHK1 levels, we hypothesise that BRCA2 protein might perform a role similar to BRCA1, via its interaction with CHK1 protein, thus facilitating maintenance of CHK1 phosphorylation during replication stress (Fig. 6B). This model entails that the loss of BRCA2 protein function should result in depletion of pCHK1 level during replication stress, which is what we observe in HU-mediated replication stress paradigm (Fig. 2C–E). The observed avoidance of S-phase checkpoint in BRCA2 deficient cells in spite of high DNA damages following replication stress implied that these cells manoeuvre through cell cycle via dysregulation of checkpoint control. Thus, we propose that BRCA2-CHK1 interaction might be responsible for cell cycle arrest in the S-phase (Fig. 3C and D) and subsequent delay in the replication restart step of BRCA2 positive cells, due to reduced origin firing compared to BRCA2 deficient cells (Fig. 2C–E), post removal of replication stress (Fig. 2F).

Although the reported activities of BRCA2 vis-à-vis RAD52 look disparate: the former protein governs fork protection against Mre11 activity (Schlacher et al., 2011) and regulates origin firing, and the latter has been implicated in break induced replication repair and fork restart during replication stress (Bhowmick et al., 2016; Sotiriou et al., 2016), our model brings home the functional equivalence between the two proteins in the context of replication stress repair. Overexpression of RAD52 in BRCA2 deficient cells restored pCHK1 level in HU treated cells (Fig. 5A and B) followed by rescue of the checkpoint arrest (Fig. 5C and D) as well as normal frequency of replication origin firing (Fig. 4A–C). Interestingly, we also found that pCHK1 interacts with RAD52 protein (Fig. 5E), which even though is a novel result in the context of mammalian cells; it has already been alluded to in a previously reported study in which a Rad52 homolog of fission yeast, Rad22 has been shown to co-localise with CHK1 protein on HO endonuclease induction (Brough et al., 2012). This interaction is very significant in the context of the observed rescue of pCHK1 defect in BRCA2 deficient RAD52 stables.

We propose that similar to BRCA1 protein (Yarden et al., 2002;

Draga et al., 2015), BRCA2 and RAD52 proteins interact with CHK1, thereby facilitating its activation and even its stable maintenance post-replication stress (Fig. 6B). Our study further shows more direct involvement of RAD52 in replication stress repair via CHK1 regulation. Several post translational modifications on RAD52 protein have already been implicated during replication stress such as phosphorylation by ATR (Sotiriou et al., 2016) or stress activated protein kinases (SAPK) (Bellini et al., 2012; Yata et al., 2014) and sumoylation (Barlow and Rothstein, 2010). In addition, Wray et al. 2014 have suggested that methylation of RAD52 stabilises the protein, recruiting it to the fork on induction of HU stress, indicating that it has a role to play in replication fork arrest.

Towards the end, we draw attention to the observed dominant negative effect of RAD52 protein on HR in BRCA2 proficient cells (Fig. 1D) and increased amount of DNA breaks on induction of replication stress (Fig. S5), that is consistent with the model that RAD52 protein is known to be involved in D-loop formation which is acted upon by MUS81 endonuclease, giving rise to DNA breaks even constitutively, without DNA damage induction (Murfuni et al., 2013). In conclusion, our study has provided a novel insight of RAD52 function in HR and replication stress via checkpoint regulation. It is possible that chemotherapy resistant tumour cells might be employing a mechanism similar to what we have proposed here, thereby underscoring the need to target RAD52 functions more effectively for better therapeutic outcomes in cancer therapy.

The basal replication stress levels are highly variable across cells which sets the initial mechanistic basis for study and therefore we would want to limit our results to the current cell-line and not extend it across cell-lines (Letessier et al., 2011) that we have not tested. A sequel to the current study must involve analyses of the proposed model across various cell types and test the general validity of the proposal. We believe that uncovering the cell-line specific differences and their physiological implications is a separate and interesting study in itself.

4. Materials and methods

4.1. Cell line, plasmids and transfections

EUFA423 and HEK293 cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal bovine serum (FBS), 20 mM Hepes, 100 µg/ml streptomycin, and 100 U/mL penicillin (Sigma). EUFA423 cells stably complemented with BRCA2 were cultured in complete DMEM containing 750 µg/ml G418. All plasmid transfections were performed using Lipofectamine 2000 (Invitrogen) as per manufacturer's recommendations. EUFA423 cells stably overexpressed with RAD52 tagged to DsRed were maintained in complete medium containing 750 ug/mL G418. siRNA against BRCA2 was purchased from Dharmacon and loss of BRCA2 protein expression was confirmed by western blotting using anti BRCA2 Ab (Calbiochem OP-95).

4.2. GST pull down assay

The 6X his tagged hRAD51, hRAD52 and UVI31+ transformed *E. coli* BL21(DE3) cells were grown at 37 °C in 25 ml LB containing 100 µg/ml ampicillin, induced at A_{600} 0.6 with 1 mM IPTG and pelleted 4 h post induction. Similarly GST tagged p53 transformed E.coli BL21(DE3)cells were cultured in 150 ml LB containing 50 µg/ml kanamycin at 37 °C, induced at A_{600} 0.6 with 1 mM IPTG and pelleted 4 h post induction. The GST-p53 overexpressing cells were lysed by sonication, centrifuged and the supernatant was incubated for binding with the 1.5 ml of Glutathione S beads (GE healthcare). The 6X his tagged hRAD51, hRAD52 and UVI31+ expressing *E. coli* BL21(DE3) cells pellets were lysed, supernatants were incubated with Ni-NTA beads (Qiagen) and the respective 6X his-tagged proteins were eluted using imidazole after washes. The proteins were dialysed against TNT buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl and 0.05% Tween 20) overnight at 4 °C. The protein concentrations were estimated using BCA assay kit (Thermofisher scientific).

Equal amount of protein (10 nMoles) was incubated with GS beads, with and without bound GST-p53 in TNT buffer (100 mM Tris-HCl pH 8, 150 mM NaCl, 0.05% Tween 20) at 4 °C for 1 h. The beads were collected and washed thrice using TNT buffer at 4 °C for 15 min each. Beads were boiled in 1X SDS-PAGE gel loading buffer at 100 °C for 10 min and centrifuged. The supernatant was electrophoresed on 12% SDS PAGE gel, transferred and western blotted using 1:3000 Mouse anti His (Sigma Aldrich) and 1:2000 anti-mouse (Roche diagnostics) antibody. Similarly, various deletion constructs of RAD52 containing 6X His-tag were pulled down using GST-p53 on GS beads and western blotted using anti-His antibody.

4.3. Co-Immunoprecipitation assay

Cells were harvested and the lysate was centrifuged at 4000 *g* for 3 min to pellet cell debris. After discarding the pellet, the lysate was centrifuged at 2000 *g* for 20 min and the pellet discarded. The pre-clear lysate was mixed with 50ul sepharose bead slurry and incubated for 30 min (4 °C) at 8 rpm. The lysate is centrifuged at 2200 *g* for 3 min to pellet the beads and the supernatant is transferred to a fresh tube, making it the starting material for immunoprecipitation. The IP sample was incubated with the desired antibody overnight. The beads are re-suspended in 1 ml PBST and transferred to a fresh tube. The centrifuged pelleted beads (2300 *g* for 20 s) are washed twice more with PBST and finally with PBS.

The beads are re-suspended in 100ul Soft Elution Buffer (0.2% (w/V) SDS, 0.1% (V/V) Tween-20, 50 mM Tris-HCl, pH = 8.0) and incubated for 7 min at 25 °C, shaking at 1000 rpm. The beads are centrifuged at 2300 *g* for 20 s and the supernatant is transferred to a fresh tube. The beads are again centrifuged and the remaining supernatant is pooled with the previous eluate. The eluate is centrifuged at 16,000 *g* for 1 min to pellet carried over beads and the supernatant (~200ul) is transferred to a fresh tube. 1 ml chilled acetone (-20 °C) is mixed to the eluate and incubated at −20 °C for 3–4 hours to precipitate the protein. The sample is centrifuged at 10,000 *g* for 5 min at 4 °C and the supernatant is discarded. Air-dry the pellet and re-suspend it in Lamelli Buffer. Proteins were separated by SDS-PAGE and visualised by western blotting. Mouse Anti-IgG and Rabbit Anti-IgG were used according to manufacturer's instructions (Sigma Aldrich).

4.4. Western blotting

Whole cell extracts were prepared by lysing cells in radioimmunoprecipitation buffer (150 mM NaCl, 50 mM Tris (pH8.0), 5 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS). Protein concentrations were measured using BCA reagent system (ThermoFischer). Equal amount of protein (~20 µg) was loaded into 10% gels, subjected to SDS PAGE, transferred onto a Nitrocellulose membrane and blocked with 5% BSA-TBST (50 mM Tris (pH7.5), 150 mM NaCl, 0.1% Tween20) for 1 h at room temperature. Immuno-detection of the proteins was performed using the following antibodies: BRCA2 (Calbiochem OP-95), pCHK1 (Ser345) (Ab 58,567), pCHK1 (Ser345) (sc-21866), CHK1 (CST 2G1D5), Actin (Abcam 8227), RAD52 (sc-H300), p53 (CST-2828S; CST-1C12). Goat anti-mouse IgG (heavy and light chain (H&L)) and goat anti-rabbit IgG (H&L) labelled with HRP conjugate were used for secondary antibodies. Bands were detected using the ECL chemiluminescence detection method (Amersham Biosciences and Bio-Rad) with exposure on X-ray film and chemiluminescence machine (Amersham)

4.5. HR assay

The pDR-GFP plasmid was stably integrated into HEK293. The HEK293 cells were transfected with pDR-GFP using lipofectamine and

single clones were picked up and screened with 1 μg/ml puromycin for 20 days to attain stable clones. Stables were divided into two sets, and each was transfected with BRCA2 siRNA 2 and negative control siRNA respectively using lipofectamine as previously explained. After 48 of double siRNA transfection, the cells were transfected with pDsRed-RAD52 and pDsRed empty vector separately as a control. Finally, after 18 h, the cells were transfected with pCBASce (I-*Sce*I endonuclease expression vector), with or without p53 expression vector (pBABE-p53). Post 48 h of pCBASce transfection, the cells were trypsinized, washed and resuspended in 38 mM sodium citrate for FACS analysis. The cells were analyzed in BD LSR Fortessa cell analyzer. Cells were gated by forward and side scatter, and fluorescence of minimum of 1×10^5 gated cells was analyzed on the FL1 (Green) and FL2 (Red) channels. GFP + cells are determined from the FL1 shift from the majority of negative population.

4.6. DNA Fiber assay

DNA Fiber Assay was performed as previously described (Kim et al., 2014; Schwab and Niedzwiedz, 2011; Zeman and Cimprich, 2015). Proliferating cells were incubated with 100 μM Chlorodeoxyuridine (CldU) and 250 μM Iododeoxyuridine (IdU) for 20 and 40 min respectively (Fig. 2A). Fiber immunostaining required treatment with 2.5 N HCl for 30 min followed by washing and blocking with 5% BSA solution in PBS for 1 h. Primary antibodies: Rat anti-BrdU (1:150) (AbD Serotec) to detect CldU and Mouse anti-BrdU (1:500) (BD Biosciences) to detect IdU, were diluted in 5% BSA in PBST and incubated for 1 h at room temperature. Secondary antibodies: Rabbit Anti-Rat Alexa 488 and Goat Anti-Mouse Alexa 596 (Molecular Probes, Invitrogen). After subsequent round of washes and blocking, another round of antibody staining was performed using Donkey Anti-Rabbit Alexa488 and Donkey Anti-Goat Alexa596. Fibers were visualised under 100X oil immersion lens, using Axiovert 200 M microscope (Carl Zeiss) equipped with Axiocam MR3 camera (Carl Zeiss). Images were captured using Axiovision Software and were analysed using CASA software, developed by Prof. Paul Chastain. Fiber lengths were measures using the ImageJ software.

4.7. EdU incorportation assay

Proliferating cells were incubated in medium containing 10 μM EdU. The experiment was carried out using ThermoFisher Scientific Click-iT™ EdU Alexa Fluor™ 488 Imaging Kit.

4.8. Flow cytometry

Cells were harvested, fixed in ice cold 70% ethanol and stored at -20 °C for atleast 12 h. Following this, cells were washed with 1X PBS to remove ethanol and stained with propidium iodide (10 μg/ml) in 38 mM sodium citrate solution containing RNase A (10 μg/ml). Flow cytometry was performed using BD Fortessa and analysis was performed using FlowJo software.

4.9. Immunofluorescence analysis

Cells were fixed using 4% paraformaldehyde (PFA) for 10 min, and permeabilized using 0.1% Triton X-100 for 15 min at room temperature. Primary antibodies were diluted in 1% BSA and coverslips having fixed and permeabilized cells were incubated in a humid chamber overnight at 4 °C. Primary antibodies used were pATM (Ser1981), pRPA32 (Abcam), γH2AX, pATR (Cell Signalling Technology). Secondary antibodies (Alexa Fluors, Thermo Scientific) were used to incubate for an hour at room temperature before mounting in counterstain DAPI (Vectashield, Vector Labs) mounting medium. Confocal images were acquired using Zeiss LSM 510 Meta Confocal Microscope and Fiji (ImageJ) was used for image processing.

4.10. Statistical analysis

Samples were analysed for test of significance using Student's T-test. Error bars were plotted using SEM. The difference between two samples was considered statistically significant if (*) $P < 0.05$, (**) if $P < 0.01$, (***) if $P < 0.001$.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biocel.2018.12.013.

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