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Cyclin D1 promotes BRCA2-Rad51 interaction by restricting cyclin A/B-dependent BRCA2 phosphorylation

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Abstract

BRCA2 plays an important role in the maintenance of genome stability by interacting with RAD51 recombinase through its C-terminal domain. This interaction is abrogated by cyclin A-CDK2-mediated phosphorylation of BRCA2 at serine 3291 (Ser3291). Recently, we showed that cyclin D1 facilitates RAD51 recruitment to BRCA2-containing DNA repair foci, and that downregulation of cyclin D1 leads to inefficient homologous-mediated DNA repair. Here, we demonstrate that cyclin D1, via amino acids 20–90, interacts with the C-terminal domain of BRCA2, and that this interaction is increased in response to DNA damage. Interestingly, CDK4–cyclin D1 does not phosphorylate Ser3291. Instead, cyclin D1 bars cyclin A from the C-terminus of BRCA2, prevents cyclin A-CDK2 dependent Ser3291 phosphorylation, and facilitates RAD51 binding to the C-terminal domain of BRCA2. These findings indicate that interplay between cyclin D1 and other cyclins such as cyclin A regulates DNA integrity through RAD51 interaction with the BRCA2 C-terminal domain.

Keywords

cyclin D1; CDK2; RAD51; BRCA2; Ser3291; homologous-mediated recombination

Introduction

Breast cancer early onset 2 (BRCA2) protein functions as a tumor suppressor that maintains chromosome integrity, and its deregulation by genetic mutations has been directly linked to tumorigenesis (1, 2). Tumors containing BRCA2 mutants exhibit elevated genomic instability and genetic mutations (3–5). Several studies established that BRCA2 plays a role in homologous recombination (HR)-mediated DNA repair (6–8). A key function of BRCA2

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Conflict of interest

The authors declare no conflict of interest.

is to mediate loading of RAD51 molecule to single stranded DNA (ssDNA) (9–11). RAD51 is a recombinase that catalyzes homologous pairing and strand exchange, thus is a central protein that controls HR (12). A recent study showed that BRCA2 also has a novel function in protecting nascent DNA in the stalled replication fork (gaps of ssDNA) from the endonuclease activity of MRE11, by mediating assembly of RAD51 onto the ssDNA (resected ends of DNA double-stranded breaks, or replication gaps) (13). BRCA2 accumulates RAD51 molecules on its RAD51-binding motifs, which are located at two areas on BRCA2: the BRC repeat domain at the middle portion, and a conserved C-terminal domain. The ability of BRCA2 to accumulate RAD51 molecules correlates with its functions. Clinically, BRCA2 mutations are predominantly detected at the C-terminal RAD51 binding domain. C-terminus mutants, such as *BRCA2 6174delT* and *6158insT* (found in human pancreatic, breast, or ovarian cancer), which lack the functional RAD51-binding C-terminal domain, exhibited reduced capacity to recruit RAD51 to DNA damage foci and limited DNA repair function (14–17). Because of the significance of it, the interaction between RAD51 and BRCA2 C-terminus is subjected to regulation.

A close relationship between DNA repair and cell division has been recognized. It is established that the mode of repair for damaged DNA is primarily determined by the phase of the cell cycle; HR repair is predominant in S to G2 phase when sister chromatid is available as a template for the repair, while non-homologous end joining (NHEJ) is the main mode of repair during G0/1 phases of the cell cycle (18). Several reports indicated that cell cycle regulatory proteins directly control proteins in DNA repair pathways. Proteins in the HR pathway are substrates for CDKs, including CtIP/SAE2 (19–22), NBS1 (22), and BRCA2 (23, 24), underlining the direct role of cell cycle proteins in the DNA repair process. Cyclin A-CDK2 (or cyclin B-CDK1) was shown to phosphorylate BRCA2 at Ser3291 in its C-terminal RAD51 binding domain. This phosphorylation event inhibits RAD51 binding to this domain, thus suppressing HR (23). The phosphorylation is believed to keep activities of RAD51, and thus HR in check when repair is not required (23). On the other hand, when DNA damage occurs, this phosphorylation is dramatically downregulated (23), thereby allowing RAD51 recruitment and initiating HR repair.

Cyclin D1 is a putative cancer-causing protein. Overexpression of cyclin D1 is detected in several human cancers, such as breast cancer (25–27), mantle cell lymphoma (28, 29), squamous cell carcinoma (30–32), and colon cancer (26, 33), where it is believed to drive cancer cell division and confer chemotherapeutic resistance (34). Recently, we and others have discovered a novel function of cyclin D1 in HR (35–37). Cyclin D1 expression facilitates RAD51 recruitment to DNA damage foci (35, 36, 38). *In vivo*, cyclin D1 is detected in RAD51-containing DNA damage sites (35). Cyclin D1 depletion by RNAi or gene targeting resulted in reduced RAD51 recruitment to the damaged foci, compromised HR efficiency, and conferred cancer cell hypersensitivity to chemotherapeutic agents such as camptothecin and etoposide, as well as to gamma irradiation (35). Cyclin D1 interacts with RAD51 directly via amino acids 90–155 (35). Interestingly, depletion of cyclin D1 by RNAi did not disrupt BRCA2 recruitment to DNA damage foci. Altogether, these findings suggested that cyclin D1 facilitates RAD51 recruitment to BRCA2-bound DNA damage foci (38). However, how cyclin D1 enhances binding between RAD51 and BRCA2 remains

elusive. Here, we focused on elucidating the mechanism by which cyclin D1 promotes the interaction between RAD51 and BRCA2.

Results

Interaction between cyclin D1 and the C-terminal RAD51-binding domain of BRCA2

Previously, using immunoprecipitation coupled with mass-spectrometry, we identified BRCA2 as a cyclin D1-interacting protein (35). We also determined by *in vitro* binding assay that cyclin D1 directly interacts with BRCA2. Analyses using fragments of BRCA2 showed that cyclin D1 interaction with BRCA2 is mediated through the most N-terminus domain of BRCA2 (B2-1, Figure 1a), and through two other areas at the C-terminus domain: amino acids 2438–2824 (B2-7, Figure 1a), and 3189–3418 (B2-9, Figure 1a) (35, 39). To further investigate these interactions, we incubated each of the purified GST-BRCA2 fragments (B2-1, B2-7, and B2-9) with cell lysates prepared from human cervical carcinoma HeLa cells. In accordance with the previous *in vitro* binding assay result, we found that endogenous cyclin D1 weakly co-precipitated with the C-terminal domains B2-7, and at a higher level with the most C-terminal domain B2-9 (Figure 1b). However, unlike the previous *in vitro* GST-binding results (35), endogenous cyclin D1 did not co-precipitated with the N-terminal domain of BRCA2 (B2-1) (Figure 1b). The interactions were verified in another cancer cell line, MCF7 (Supplementary Figure S1A). These results indicated that endogenous cyclin D1 primarily interacts with the C-terminus of BRCA2 (B2-7, -9).

To investigate the interaction between cyclin D1 and the C-terminal BRCA2 domain during the cell cycle, we prepared cell lysates from HeLa cells synchronized in G1, S, G2-M phase and verified expressions of cyclin D1, A, and B. We verified that cyclin D1 expression was high in G1 phase, and gradually decreased when cells entering S, then G2-M. Cyclin A expression peaked in S-phase, while cyclin B upregulated during late S and G2-M (Figure 1c). We then incubated B2-9 fragment in the lysates. We found that endogenous cyclin D1 co-precipitated with the C-terminal fragments of BRCA2 (B2-9) from lysates prepared from cells in G1, S, and G2-M phase (Figure 1d). Despite high cyclin D1 expression in G1, and lower cyclin D1 expression in S and G2-M phase, we detected an interaction between cyclin D1 and B2-9 in every phase of the cell cycle, with slightly stronger interactions in S, and G2-M-phase. We then performed immunoprecipitation using an antibody that recognizes endogenous BRCA2 in lysates prepared from HeLa cells, followed by immunoblotting to detect co-precipitated cyclin D1. We found that endogenous cyclin D1 interacted with the endogenous BRCA2 (Supplementary Figure S1B). Consistently, the interaction between endogenous cyclin D1 and BRCA2 was weaker in cells synchronized in G1, and was upregulated in cells in S and G2-M-phase (Supplementary Figure S1B), implying that the affinity of cyclin D1 towards BRCA2 may be regulated during the cell cycle. We also found that endogenous cyclin A interacted with the C-terminal BRCA2 fragment B2-9, in every phase of the cell cycle (Figure 1d). The interaction was slightly upregulated in S and G2-M phase. Endogenous cyclin B marginally interacted with the B2-9, and was only upregulated in the G2-M. These observations indicated that various cyclins interact with BRCA2 at the C-terminal domain B2-9. The differential interactions between cyclin D1 and BRCA2 fragments during each phase of the cell cycle suggested that the interactions are specific and

may be regulated. A previous report, showed that cyclin A-B2-9 interaction relies on Cy or RXL motif on BRCA2 (23). We found that the interaction between cyclin D1-B2-9 was independent of the RXL sequence, since the Cy peptide inhibitor did not interfere with the interaction (Supplementary Figure S1C).

Cyclin D1 prevents BRCA2 Ser3291 phosphorylation by CDKs

RAD51 was shown to directly interact with the end-most C-terminal fragment of BRCA2 (B2-9) (9, 40, 41). The interaction between RAD51 and B2-9 is abrogated by the phosphorylation of BRCA2 at Ser3291 mediated by cyclin A-CDK2, or when Ser3291 was mutated to glutamic acid (S→E, a phospho-mimicking mutation/ S3291E mutant) (23).

Conversely, the interaction between RAD51 and the C-terminus fragment of BRCA2 was enhanced when Ser3291 phosphorylation was blocked by a CDK2/1 chemical inhibitor, roscovitine (23). These data demonstrated that binding of RAD51 to the C-terminus of BRCA2 is negatively controlled by kinase activity of the cell cycle protein, CDK2/1, and prevention of this phosphorylation event enhances RAD51 recruitment to the C-terminus of BRCA2 (23).

To elucidate the mechanism by which cyclin D1 facilitates interaction between RAD51 and BRCA2, we focused on the interaction between cyclin D1 and the B2-9 fragment of BRCA2 for the following reasons. First, the interactions of cyclin D1–BRCA2 and of RAD51–BRCA2 are specific to the B2-9 fragment. In line with this observation, our previous results indicated that a physical interaction between cyclin D1 and RAD51 is required for HR (35). Second, as we showed here, various cyclins interact specifically with B2-9, suggesting a degree of interplay among these proteins at this BRCA2 domain. Lastly, some of these cyclins, particularly cyclin A, was implicated to be important regulators of RAD51 binding to this domain(23).

Because the phosphorylation of Ser3291 was shown to be a critical factor that determines RAD51 binding to the C-terminus of BRCA2, and it was associated with cyclin A or cyclin B expression (23), we examined whether cyclin D1 overexpression is associated with Ser3291 hyperphosphorylation.

BRCA2 phosphorylation at Ser3291 was clearly detected by a specific antibody (23) in lysate prepared from asynchronous HeLa cells (Figure 2a, lane 1). As previously reported (23), Ser3291 phosphorylation was highly upregulated when cells were synchronized in early mitosis (prometaphase) by nocodazole treatment (Figure 2a lane 4), and was completely suppressed by roscovitine treatment, confirming that this is CDK2/1-dependent phosphorylation (Figure 2a, lane 3 and 6). Interestingly, we found that overexpression of cyclin D1 did not increase phosphorylation at Ser3291; instead, it significantly suppressed the phosphorylation (Figure 2a, lane 2 and 5). Overexpression of cyclin D1 neither affected the expression of cyclin D-dependent kinase 4 (CDK4), BRCA2, and RAD51 protein, nor disturbed the cell cycle distribution of the cells (Figure 2a, b). In agreement with this, cyclin D1 depletion by cyclin D1-specific short-interfering RNAs (siRNAs) enhanced BRCA2 phosphorylation at Ser3291 (Figure 2c).

Given that some CDKs share a common substrate, we investigated if cyclin D1-CDK4 phosphorylates B2-9. We performed *in vitro* cyclin D1-CDK4 and cyclin A-CDK2 kinase assays on purified C-terminal domain GST-B2-9. In accordance with a previous report (23), cyclin A-CDK2 phosphorylated B2-9, but not B2-5 (B2-5 was used as a negative control) (Supplementary Figure S2). In contrast, although the cyclin D1-CDK4 exhibited strong kinase activity toward a C-terminal fragment of pRB (used as a positive control), phosphorylation of GST-B2-9 by cyclin D1-CDK4 was undetectable (Supplementary Figure S2). Therefore, we concluded that the C-terminal fragment of BRCA2 (B2-9), while a suitable substrate for cyclin A-CDK2, is not a substrate for cyclin D1 and its associated kinase partner CDK4.

To study the role of cyclin D1 on BRCA2 phosphorylation at Ser3291 *in vivo*, we depleted cyclin D1 expression from HeLa cells using a short hairpin RNA (shRNA) specific to cyclin D1 (35). We then synchronized the cells in late G1 and released them to re-enter the cell cycle. Ser3291 phosphorylation and expression of cyclins were analyzed by immunoblotting using specific antibodies (Figure 2d). HeLa cells do not contain functional pRB, therefore, expression of cyclin D1 is not required for proliferation of these cells (42, 43). Accordingly, depletion of cyclin D1 did not alter the cell cycle profiles of these cells (Supplementary Figure S3A and B). In control cells expressing non-target shRNA, we found that BRCA2 Ser3291 phosphorylation was low during G1 to S-phase (at 0, 1, 2, 3 hrs after release), upregulated when most cells were leaving S and entering G2 (4 hr). The phosphorylation then declined when cells started to leave G2 to enter G1 (at 5, and 6 hrs). The upregulation of Ser3291 phosphorylation correlated with elevated expression of cyclin A and cyclin B, and the downregulation of the Ser3291 correlated with high level of cyclin D1 expression (Figure 2d). In cyclin D1-depleted cells, Ser3291 phosphorylation was upregulated during G1 and S phase (0, 1, 2, 3 hrs), and at the late G2-M (5, 6 hrs). As a result, Ser3291 was hyperphosphorylation throughout cell cycle. We also noticed early upregulation of cyclin A. Hence, Ser3291 phosphorylation is influenced by the relative expressions of cyclin D1/A in the continuously growing cell.

Cyclin D1 expression inhibits binding of cyclin A to the C-terminus of BRCA2 and promotes RAD51 binding

We then investigated the effect of cyclin D1 expression on the interaction between RAD51 and the BRCA2 C-terminal domain. To this end, we incubated purified C-terminal BRCA2 B2-9 fragment in cell lysates prepared from HeLa cells in a buffer with a high ATP. The proteins co-precipitated with the fragment were analyzed using specific antibodies. After incubation, B2-9 was efficiently phosphorylated at Ser3291, as it was detected by the phospho-Ser3291 BRCA2-specific antibody (Figure 3, lane 2). Under this condition, the Ser3291 phosphorylated B2-9 fragment co-precipitated with cyclin A and a small amount of RAD51 (Figure 3, lane 2). When incubated in lysate prepared from cells treated with roscovitine, phosphorylation at Ser3291 on B2-9 was significantly suppressed (Figure 3, lane 4). Inhibition of Ser3291 phosphorylation by roscovitine was associated with increasing amounts of RAD51 co-precipitated with B2-9 (Figure 3; lane 4 compared with lane 2).

When incubated in lysates prepared from cells ectopically expressing cyclin D1, Ser3291 phosphorylation on B2-9 became virtually undetectable (Figure 3, lane 3). Under this condition, we observed that the B2-9 interaction with RAD51 was greatly enhanced, while the interaction with cyclin A was significantly reduced (Figure 3, lane 3). We also observed that cyclin D1 clearly co-precipitated with the fragment (Figure 3, lane 3).

The C-terminal domain of BRCA2 preferentially binds to cyclin D1 over cyclin A

As both cyclin D1 and cyclin A are capable of binding to the C-terminal domain of BRCA2 (B2-9), we compared the affinities of both proteins toward the C-terminal fragment of BRCA2. Increasing amounts of cyclin A or cyclin D1 were added to the *in vitro* binding assay reactions, which were composed of purified HA-tagged-cyclin D1 and GST-B2-9.

Compared to cyclin D1, cyclin A was a weaker competitor for B2-9 binding (Figure 4a, b). The concentration of purified cyclin A that dislodged 50% of HA–cyclin D1 from B2-9 was 28.5 nM, while that of purified cyclin D1 was 11.2 nM (Figure 4a, b).

In a reverse experiment, in which purified cyclin D1 and cyclin A competed against HA–cyclin A for B2-9 binding, we confirmed that cyclin D1 was a stronger competitor than cyclin A for binding to B2-9. The concentration of purified cyclin D1 required to dislodge HA–cyclin A was 9.5 nM, while that of purified cyclin A was 29.5 nM (Supplementary Figure S4). Therefore, cyclin D1 is a preferred cyclin partner over cyclin A for the C-terminus of BRCA2.

Cyclin D1 and DNA damage cooperate to suppress Ser3291 phosphorylation

Ser3291 phosphorylation is an important regulatory event that restricts RAD51 recruitment to the C-terminal domain of BRCA2, and thus suppresses HR DNA repair (23). DNA damage was demonstrated to suppress phosphorylation at this moiety (23) (Figure 5a). Upon subjection to ionizing radiation (IR), we found that binding of cyclin A to the C-terminus BRCA2 fragment was significantly reduced (Figure 5a). Interestingly, IR treatment significantly enhanced binding of cyclin D1 to the C-terminal B2-9 fragment of BRCA2 (Figure 5a). We then analyzed Ser3291 phosphorylation on endogenous BRCA2 by immunoblotting. Again, we found that nocodazole treatment in HeLa cells enriched cells in G2/M phase and enhanced BRCA2 Ser3291 phosphorylation to a level that was much higher than that of untreated cells (Supplementary Figure S5A, and Figure 5b, lane 2 compared with lane 1). Roscovitine suppressed Ser3291 phosphorylation, confirming that this phosphorylation was cyclin A/B-CDK2/1-dependent phosphorylation (Figure 5b, lanes 3, 4). Ectopic expression of cyclin D1 or DNA damage suppressed Ser3291 phosphorylation in both nocodazole-treated and -untreated cells (Figure 5b, lanes 5, 6 compared to lanes 1, 2, and lanes 7, 8, compared to lanes 1, 2). Cyclin D1 overexpression and IR treatment suppressed BRCA2 Ser3291 phosphorylation completely, both in untreated and nocodazole-treated cells (Figure 5b, lane 9, 10). Of note, ectopic expression of cyclin D1 did not change cell cycle profiles of HeLa cells (Supplementary Figure S5A). To understand how DNA damage increases cyclin D1 binding to BRCA2, we analyzed levels of BRCA2 Ser3291 phosphorylation and CDK2 activity at various time points after DNA damage. Under the moderate DNA damaging condition (5 Gy IR), BRCA2 Ser3291 phosphorylation decreased

at 30 min after DNA damage (Figure 5c). This correlated well with decreasing CDK2 kinase activity on BRCA2 Ser3291 (Supplementary Figure S5B). At this time point, cyclin D1 interaction with B2-9 was increased (Supplementary Figure S5C). Of note, we found that expressions of cyclin D1 and A were still unchanged at 0.5 hr (data not shown). Previous reports have shown that reduction of CDK2 activity after DNA damage is caused by rapid destruction of CDC25A in response to DNA damage (44, 45). In agreement with that, we found a rapid degradation of CDC25A at 30 after IR (Figure 5c). To investigate whether cyclin D1 can bind to BRCA2 C-terminus, when Ser3291 is phosphorylated, we pulled down cyclin D1 from cell lysate using the phospho-mimicking form of B2-9 (S3291E). We found that cyclin D1 bound modestly to the B2-9 S3291E, when compared to B2-9 (Figure 5d). These results pointed out that, after DNA damage, CDK2/1 activity rapidly declines, resulting in decreased BRCA2 Ser3291 phosphorylation. During this time, cyclin D1 rapidly occupies the regulatory region C-terminus of BRCA2, thus no longer allows further Ser3291 phosphorylation.

To investigate the possibility that cyclin D1 also directly enhances RAD51 recruitment to B2-9, we performed *in vitro* binding assays between RAD51 and the C-terminus of BRCA2 in the presence of cyclin D1. Purified RAD51 specifically bound to the C-terminus B2-9 fragment of BRCA2 in the presence or absence of cyclin D1, indicating that cyclin D1 is not required for recruitment of RAD51 to B2-9 (Supplementary Figure S6, lane 6-10). Increasing the amount of cyclin D1 in the reaction gradually increased cyclin D1 binding to B2-9 (Supplementary Figure S6, lanes 7-10). However, the increased levels of purified cyclin D1 did not enhance the recruitment of RAD51 to the C-terminus of BRCA2 (Supplementary Figure S6, lanes 7-10).

These results indicated that cyclin D1 does not directly recruit RAD51 to the C-terminus of BRCA2. Therefore, the role of cyclin D1 in RAD51 recruitment is plausibly to prevent the inhibitory Ser3291 phosphorylation mediated by other cyclins.

Amino acids 20–90 at the N-terminus of cyclin D1 are required for binding to the C-terminus of BRCA2

To identify the BRCA2 binding domain of cyclin D1, we constructed two cyclin D1 truncated mutants; cyclin D1 1–19 that lacks amino acids 1–19 at the N-terminus of cyclin D1, and cyclin D1 1–90 that lacks amino acids 1–90 (Figure 6a). We tested the mutants in an *in vitro* binding assay. We found that purified full-length cyclin D1 and cyclin D1 1–19 were able to interact with the B2-9 fragment of BRCA2 (Figure 6b, lane 2, 3), therefore amino acids 1–19 of cyclin D1 were not required for binding to the C-terminus of BRCA2. The mutant cyclin D1 1–90 no longer interacted with the C-terminus of BRCA2, which indicated that the interaction between cyclin D1 and the C-terminal domain of BRCA2 is mediated through amino acids 20–90 of cyclin D1 (Figure 6b lane 4). In accordance with this, while purified full-length cyclin D1 prevented B2-9 phosphorylation caused by cyclin A-CDK2 in an *in vitro* kinase assay, mutant cyclin D1 1–90 did not prevent phosphorylation as efficiently as the full-length protein (Figure 6c, d).

We also investigated effect of cyclin D1 C-terminus modification, specifically threonine 286 phosphorylation, on the BRCA2 interaction. We found that expression of oncogenic

phosphodegron mutant cyclin D1 T286A, which is defective in phosphorylation-mediated nuclear export and subsequent proteolysis (46–48), was able to suppress BRCA2 Ser3291 phosphorylation. The mutant cyclin D1 bound to BRCA2 B2-9, and facilitated HR repair (Supplementary Figure S7A–C), suggesting that post translation modification at T286 is not required for cyclin D1-BRCA2 interaction.

Discussion

Well-controlled phosphorylation of BRCA2 is required for the BRCA2-dependent genome maintenance (23, 24, 39, 49, 50). Recent evidences indicated that alteration of the Ser3291 phosphorylation leads to attenuated RAD51 recruitment and BRCA2 function loss, which associated with genome instability (23, 50). Previously, we identified cyclin D1 as an important protein required for RAD51 recruitment to BRCA2-positive DNA repair foci and efficient HR repair (35). Here, we elucidated a possible mechanism employed by cyclin D1 to promote the recruitment of RAD51 to BRCA2. We found that overexpression of cyclin D1 in the absence of DNA damage was effective enough to suppress cyclin A/B-CDK2/1-dependent BRCA2 Ser3291 phosphorylation. This may be explained by the higher affinity of cyclin D1 toward BRCA2 C-terminus, compared to that of cyclin A. Via amino acids 20–90, cyclin D1 interacts directly with the C-terminus of BRCA2 at amino acids 3189–3418, and impedes the inhibitory CDK2/1-dependent BRCA2 Ser3291 phosphorylation. Thus, cyclin D1 does not enhance RAD51 binding to B2-9 *per se*. Instead, cyclin D1 indirectly facilitated RAD51 recruitment and HR-mediated DNA repair by fencing off the inhibitory phosphorylation caused by cyclin A/B-CDK2/1.

From our results, we propose that during normal cell cycle, levels of BRCA2 Ser3291 phosphorylation and HR are balanced by relative levels of cyclin D1 and cyclin A/B. Under DNA damage conditions, however, activity of CDKs rapidly declines, because of a rapid degradation of CDC25A, resulting in Ser3291 hypophosphorylation. Then cyclin D1, which preferably interacts with hypophosphorylated form of C-terminal domain of BRCA2, accumulates at the domain and precludes kinases, i.e. cyclin A-CDK2 complex from this site. Thus, this mechanism ensures increased HR-mediated DNA repair (Figure 7). According to this view, neither expression of cyclin D1 nor CDKs activity determine the amount of RAD51 recruitment to BRCA2 C-terminus. Rather, the equilibrium between them is a control mechanism that indicates the outcome. Recent report showed that, cells irradiated with a sub-lethal dose of IR for a long period adapted by upregulating cyclin D1 expression (51), suggested that cyclin D1 is required for DNA damage response and cell survival. Intriguingly, extended period of cyclin D1 overexpression was shown to cause genome instability (51, 52). One speculation is that, the elevated level of cyclin D1 disturbs the cyclins D1/A balance in the cells, therefore, interferes with the regulation of the Ser3291 phosphorylation and HR. In some types of cancer, for example, T-cell acute lymphoblastic leukemia (T-ALL), high level of cyclin D3 was detected (with non-detectable levels of cyclin D1 and cyclin D2) (53, 54). We found that in a T-ALL cell line Jurkat, cyclin D3 interacted with B2-9, and suppressed B2-9 Ser3291 phosphorylation (Supplementary Figure S8A–B). Cyclin D3 was also co-immunoprecipitated with endogenous BRCA2 (Supplementary Figure S8C), and was able to moderately restore HR in cyclin D1-depleted

cells (Supplementary Figure S7C). These results support the notion that D type cyclins may have a general role in preserving genome integrity.

Recently, two groups elegantly revealed that BRCA2 and RAD51 function in blocking stalled replication fork degradation caused by MRE11 (13, 55). In one study, the C-terminal RAD51-binding domain of BRCA2 was shown to be essential for this novel function (13). Whether or not cyclin D1 participates in this novel function remains to be determined.

Since, cyclin D1 works with BRCA2 and RAD51 to facilitate HR, altered cyclin D1 expressions may interfere with HR. Accordingly, we recently showed that cyclin D1 depletion sensitized cancer cells to a poly (ADP-ribose) polymerase (PARP) inhibitor treatment (35). This is in consistent with reports, that deficiency in HR renders cells hypersensitive to PARP inhibitors (56). Our results indicated that in cyclin D1-expressing cancers that contain wild-type BRCA2 protein, targeting cyclin D1 in combination with DNA-damaging agents may be beneficial for the cancer treatment.

Materials and Methods

Cell lines and synchronization

Jurkat, Granta519, HEK293, HeLa and MCF7 cells were from ATCC (Manassas, VA, USA). HEK293 DR-GFP cell line was established as described previously (35). Roscovitine and nocodazole treatments were performed as previous (23). HeLa cells were synchronized in G1 phase by lovastatin (57), in S phase by double thymidine block and release (23), and in prometaphase by 50 µg/l nocodazole (23). For cell cycle re-entry, cells were synchronized by double thymidine block (23). For cell cycle distribution analyses, cells were stained with propidium iodide and analyzed by FACS. Shown are percentages of cells in particular cell cycle phases from corresponding figures. DR-GFP assay results were analyzed from 3 independent experiments, using *Student's* t test. The results are considered significantly different when *P-value* less than 0.01.

Production of recombinant proteins and binding assays

Production of recombinant cyclin D1, cyclin A, and deleted mutants were performed according to a protocol described previously (35). Constructs encoding GST-fragments of BRCA2 (39) were kindly provided by Dr. A. Venkitaraman, University of Cambridge. GST-B2-9 S3291E was described previously (23). *In vitro* binding was performed as described (23) with some modifications. Briefly, 1 µg of each GST fusion protein was incubated (30 min, 37 °C) with 5 µl of GSH Sepharose in 200 µl binding buffer (20 mM Hepes pH 7.5, 150 mM KCl, 10% glycerol, 0.1 % NP40, 1 mM EDTA, 5 mM MgCl₂, 1 mM DTT, 0.5 mM PMSF). One hundred ng of tested proteins were added and binding reactions were incubated for another 30 min at 37 °C, followed by 1 h incubation at 4°C. After binding, beads were washed 4 times with 0.5 ml of ice-cold binding buffer. Proteins were separated using SDS-PAGE gels and analyzed by immunoblotting using cyclin D1- and GST-specific antibodies. Peptide (Cy) inhibition assay was performed as previous (23).

GST pull-down of endogenous cyclins and co-immunoprecipitation

The lysates from HeLa cells at 80% confluency were prepared in 0.5% NP40, ELB buffer (0.5% NP40, 160 mM NaCl, 50 mM HEPES, pH 7.4, 50 mM EDTA, proteinase inhibitors). One μ g of each GST fusion protein was incubated overnight at 4°C in 1 mg of lysate. GST-BRCA2 fragments were pulled down using 20 μ l of GSH Sepharose and washed with cold 0.5% ELB buffer. The pull-down products were run on SDS-PAGE gels and analyzed by immunoblotting using specific antibodies. In experiments, which phosphorylation of GST-B2-9 was to be examined, pull-down experiments were performed in kinase buffer (58) without the addition of γ -³²P ATP. Co-immunoprecipitation of endogenous BRCA2 and cyclin D1 was performed using a monoclonal antibody specific to BRCA2, and cyclin D1 immunoblotting was performed using a rabbit anti-cyclin D1 antibody. Plasmid pcDNA cyclin D1 HA T286A was from Addgene, Cambridge, MA, USA.

Cyclin D1/cyclin A competition assay

Competition assays were performed as previously described (59). Briefly, 10 nM of purified GST-B2-9 was incubated with 10 nM of HA-cyclin D1 in the binding buffer. Various amounts (0 nM, 10 nM, 20 nM, 40 nM, and 80 nM) of cyclin D1 or cyclin A were added to the reaction. BSA was used to control total protein amount in each reaction. GST-B2-9 and the interacting proteins were pulled down using 10 μ l of GSH Sepharose. The pull-down products were separated by SDS-PAGE gel electrophoresis and analyzed by immunoblotting using specific antibodies.

In vitro CDK kinase assay

CDK4 kinase reactions were performed as previously described (58). cyclin A-CDK2 kinase assays were performed similarly, except that cyclin D1-CDK4 was replaced with active cyclin A-CDK2 (EMD Millipore, Billerica, MA, USA). B2-5 was used as a substrate for the negative control. In the competition assay, increasing concentrations of recombinant proteins (cyclin D1, cyclin D1 1-90, or cyclin D1-CDK4) at 10 nM, 20 nM, or 40 nM were added to the reaction. Active CDK2 complex was immunoprecipitated by CDK2-specific antibody, then subjected to *in vitro* kinase assay.

siRNA, shRNA and antibodies

Cyclin D1-specific siRNA A (siD1-A, 5'-CCAAUAGGUGUAGGAAAUAGCGCTG-3') was from Integrated DNA Technologies. Cyclin D1-specific siRNA B (siD1-B, 5'-AACACCAGCTCCTGTGCTGCG-3'), C (siD1-C, 5'-GCCCTCGGTGTCCTACTTCAA-3'), control siRNA (AllStars Negative control) were from Qiagen (Valencia, CA, USA). Cyclin D1 shRNA (5'-GCCAGGATGATAAGTTCCTTT-3'), and non-target shRNA (5'-CAACAAGATGAAGAGCACCAA-3') were from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). The following antibodies were used: anti-cyclin D1 H295, RAD51 H-92, cyclin A C-19, GST Z-5, CDK2 M-2, CDK4 C-22 antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), cyclin D3 DCS-22, antibody against the C-terminus of cyclin D1 (Ab3, Thermo Fisher Scientific, Waltham, MA, USA), anti-BRCA2 OP-95 antibody (EMD Millipore), anti-HA 12CA5, CDC25A, γ H2AX antibodies (abcam, Cambridge, MA, USA),

anti- β actin AKR-002, GAPDH AKR-001 antibodies (Sigma-Aldrich). Anti-phospho-Ser3291 BRCA2 antibody was described previously (23).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Cyclin D1 interacts with the C-terminus of BRCA2.

a) Diagram depicting GST-BRCA2 fragments designated as B2-1, B2-2, B2-5, B2-7, B2-9 (39). The numbers adjacent to each fragment indicate the BRCA2 amino acids spanned by the fragments. Grey lines, BRC repeats; black line at the C-terminus indicates position of Ser3291. **b)** Interactions between GST-BRCA2 fragments and endogenous cyclin D1. B2-1, B2-2, B2-7, and B2-9 were incubated with lysates prepared from HeLa cells. Endogenous proteins co-precipitated with the GST-BRCA2 fragments were analyzed by immunoblotting (IB) using the indicated antibodies. GST immunoblot shows input GST-BRCA2 fragments. **c)** Immunoblotting of cyclin D1, A, and B expressions in lysates (WCL) synchronized in G1, S, and G2-M phase used in **(d)**, AS; asynchronous. GAPDH was used as a loading control. **d)** Interactions between GST-BRCA2 fragment B2-9 and cyclins in G1, S, and G2-M phase of the cell cycle. GST-BRCA2 fragment B2-9 was incubated with lysates prepared from HeLa cells synchronized in G1, S, and G2-M phase (see Materials and Methods). Co-precipitated cyclins were analyzed using specific antibodies.

Figure 2. Cyclin D1 suppresses BRCA2 Ser3291 phosphorylation

(a) Immunoblot (IB) analyses of phospho-Ser3291 BRCA2 (S3291 Ph) in HeLa cells, HeLa cells ectopically expressing cyclin D1 (D1), and HeLa cells treated with roscovicine (Ros). Lanes 1–3 contained lysates prepared from asynchronous cells, while lysates in lanes 4–6 were prepared from nocodazole treated cells. Expressions of cyclin A, B, D1, CDK4, BRCA2, and RAD51 were analyzed as indicated. Actin was used as a loading control **(b)** Cell cycle distribution of HeLa cells from **(a)** with indicated treatments. **(c)** Immunoblot (IB) analyses of phospho-Ser3291 BRCA2 (S3291 Ph) in HeLa cells treated with cyclin D1-specific small interfering RNAs (siD1). **(d)** Immunoblot (IB) analyses of phospho-Ser3291 BRCA2 (S3291 Ph), BRCA2, and cyclins during the cell cycle. Lysates were prepared from HeLa cells expressing a cyclin D1-specific short hairpin RNA (shcyclin D1), or non-target short hairpin RNA (shcont). Actin and GAPDH were used as a loading control.

Figure 3. Cyclin D1 expression inhibits binding of cyclin A to the C-terminus of BRCA2 and promotes RAD51 binding.

Immunoblot analyses of proteins co-precipitated with B2-9 under different conditions. Lane 1, B2-9 incubated with binding buffer alone; lane 2, B2-9 incubated in HeLa cell lysates.

Lane 3, B2-9 incubated in lysates prepared from HeLa cells overexpressing cyclin D1 (D1), or in cells pretreated with roscovitine (Ros) (lane 4). Co-precipitated proteins were analyzed using the specific antibodies indicated.

Figure 4. Competition between cyclin D1 and cyclin A for binding to the C-terminus of BRCA2.

a) C-terminal fragment of BRCA2 (B2-9) was pre-mixed with purified HA-cyclin D1.

Increasing amounts (0nM, 10nM, 20nM, 40nM, and 80nM) of either purified cyclin D1 or cyclin A were added to the reaction. Amounts of HA-cyclin D1 co-precipitated with B2-9 were analyzed by immunoblotting (IB) using an anti-HA antibody. GST-B2-9 inputs were analyzed by an anti-GST antibody. BSA was added into the reactions to maintain equal amount of protein in every reaction. **b)** Percentages of HA-cyclin D1 bound to B2-9 in the presence of purified cyclin D1 or cyclin A from **(a)**.

Figure 5. Cyclin D1 cooperates with DNA damage to inhibit BRCA2 phosphorylation at Ser3291.

a) Co-precipitation of cyclin A and cyclin D1 at 0.5 hr after 5 Gy IR treatment. B2-9 was incubated with HeLa cell lysates prepared from cells with (+) or without IR treatment (-). Co-precipitated proteins were analyzed by immunoblotting (IB) using specific antibodies. Phospho-Ser3291 on B2-9 was also analyzed. GST-B2-9 input was verified using a GST-specific antibody. **b)** Levels of phospho-Ser3291 (S3291 Ph) under various treatments were analyzed by immunoblotting (IB). The treatments included nocodazole, ionizing radiation (IR, 5 Gy), ectopic cyclin D1 expression (D1), and roscovitine (Ros). Expression of BRCA2, cyclin D1, γ H2AX and CDK4 were also analyzed. Actin was used as a loading control. Drug treatment was maintained for 24 hr, at which time the extracts were prepared. **c)**

Immunoblots (IB) indicate expression levels of CDC25A, BRCA2, and levels of S3291 Ph at time points after 5Gy IR treatment. Actin was used as a loading control. **d)** Interaction between cyclin D1 and the phospho-mimicking B2-9 S3291E. GST-B2-9 or B2-9 S3291 was incubated with lysates prepared from HeLa cells. Cyclin D1 co-precipitated with the GST-fragments were analyzed by immunoblotting (IB) using the indicated antibodies. GST immunoblot shows input GST-BRCA2 fragments.

Figure 6. Amino acids 20–90 of cyclin D1 are required for BRCA2 C-terminus binding.

a) Schematic diagrams of full-length (cyclin D1 1–295) and truncated mutants (Δ1–19 and Δ1–90). Light grey color highlights indicate known functional domains of cyclin D1, such as pRB binding domain, cyclin box, and pest sequence (60). **b)** *In vitro* binding assays using GST–BRCA2 B2-9 and purified full-length cyclin D1 (aa1–295) or the indicated cyclin D1 mutants. Upper panel: indicated proteins were mixed, GST-containing proteins were precipitated using GSH Sepharose, resolved by SDS-PAGE and immunoblotted (IB) with an antibody specific to the C-terminus of cyclin D1. Lower panel: blot was re-probed with an anti-GST antibody. Input cyclin D1 and mutants were verified by immunoblotting (right panel). GST–BRCA2 B2-5 was used as a non-binding negative control for pull-downs. **c)** B2-9 phosphorylation by CDK2 was efficiently inhibited by full-length cyclin D1, but not by Δ1–90 mutant. *In vitro* CDK2 kinase assays were performed with increasing amounts (0nM, 10nM, 20nM, 40nM) of either purified cyclin D1 or Δ1–90. Kinase activities were analyzed by autoradiography of ^{32}P transferred to B2-9 by cyclin A-CDK2. Immunoblotting was performed to verify levels of GST–B2-9 and purified cyclin D1 and Δ1–90, using a GST- and a cyclin D1-specific antibody. **d)** Relative densities of the signals from (c).

Figure 7. Prevention of BRCA2 Ser3291 phosphorylation by cyclin D1.

When HR-repair is not required, i.e. no DNA damage, RAD51 recruitment to BRCA2 C-terminus is precluded by CDK2/1-dependent phosphorylation at BRCA2 Ser3291 (left). However, when HR repair is required, cyclin A-CDK2-mediated phosphorylation of BRCA2 at serine 3291 is suppressed by rapid degradation of CDC25A, and by cyclin D1 hindering of cyclin A-CDK2 complex to the phosphorylation site. These conditions facilitate RAD51 recruitment to the BRCA2 C-terminus and HR repair (right).