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Proteomic identification of a direct role for cyclin D1 in DNA damage repair

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Abstract

The human *CCND1* gene, encoding a cell cycle protein cyclin D1, is one of the most frequently amplified genes in human cancers. Cyclin D1 activates cyclin dependent kinases CDK4 and CDK6 and drives cell proliferation. Beyond the cell cycle role, the full repertoire of cyclin D1 functions in cancer cells is still unclear. Emerging evidence indicates that cyclin D1 may play a role in DNA damage response. In this review we discuss observations linking cyclin D1 to DNA damage repair, and we summarize our recent findings, which demonstrate cyclin D1 function in homologous recombination-mediated DNA repair.

Cyclin D1 in cell cycle progression

D-type cyclins are components of the core cell cycle machinery. The D-cyclin family is composed of three proteins, cyclin D1, D2 and D3, which are expressed in proliferating cells (1). The gene encoding cyclin D1 represents the second most frequently amplified locus in the human cancer genome (2). The protein product of this locus, cyclin D1, binds and activates cyclin-dependent kinases CDK4 and CDK6 (1). During cell cycle progression, cyclin D1-CDK4 and D1-CDK6 complexes phosphorylate the retinoblastoma protein, pRB, pRB-related p107 and p130 proteins, as well as Smad3 and FOXM1 transcription factors (1, 3, 4). By far the best-documented function of cyclin D1 is its ability to drive cell cycle progression through phosphorylation of pRB, p107 and p130. In their hypophosphorylated forms pRB, p107 and p130 inhibit the transcriptional activity of E2F transcription factors. Phosphorylation of these three proteins by cyclin D1-CDK4/6 kinase releases and de-represses E2Fs, thereby allowing G1→S phase progression (1) (Figure 1A). In addition to this kinase-dependent function, cyclin D1-CDK4/6 complexes sequester cell cycle inhibitors $p27^{Kip1}$ and $p21^{Cip1}$ away from cyclin E-CDK2, thereby contributing to activation of cyclin E-CDK2 kinase (1). Lastly, there is growing evidence that cyclin D1 plays cell cycle-independent roles which are also independent of CDK4 and CDK6 (5).

Degradation of cyclin D1 upon DNA damage

Proliferating cells usually respond to DNA damage by arresting their cell cycle progression. Several independent reports pointed to downregulation of cyclin D1 as one of the mechanisms that underlie this cell cycle arrest (6–8). DNA damage was shown to activate GSK3β, which phosphorylates cyclin D1 on Thr-286. Phosphorylated cyclin D1 is then exported from the nucleus, polyubiquitinated by the $SCF^{Fbx4\text{-}aBcrystallin}$ E3 ubiquitin ligase, and degraded by the proteasome (7, 9). Strikingly, a related cyclin D2 does not undergo phosphorylation on the corresponding residue following DNA damage, suggesting that cyclin D1 may play a non-redundant role in transmitting post-radiation growth-arresting signals to the core cell cycle machinery (7, 9). The activity of ATM was shown to be

required for cyclin D1 phosphorylation and degradation triggered by double stranded DNA breaks, while the ATR kinase mediates the effect on cyclin D1 following UV irradiation (7, 10, 11). In contrast to these findings implicating F-box protein Fbx4 and cofactor αB crystallin in degradation of cyclin D1, another group postulated that Thr-286-phosphorylated cyclin D1 interacts with and is targeted for degradation by an F-box protein FBXO31 (8). Moreover, DNA damage was shown to cause proteolysis of cyclin D1 by the anaphase promoting complex/cyclosome (APC/C). This effect is mediated by the destruction box in cyclin D1 and it was shown to be independent of cyclin D1 phosphorylation on Thr-286 (6). It is possible that these different scenarios reflect distinct modes of cyclin D1 degradation in particular cell types. Overall, these reports point to cyclin D1 degradation as an important molecular mechanism which arrests cell proliferation following DNA damage. Persistent high expression of cyclin D1 in cells which accumulated double-stranded DNA breaks leads to radio-resistant DNA synthesis (7). Moreover, downregulation of cyclin D1 following UV damage was shown to be required for efficient DNA repair, and forced overexpression of cyclin D1 prevented DNA repair (12).

To complicate this picture, some reports documented an increase of cyclin D1 levels following DNA damage (13–16). These findings hint that cyclin D1 may play an active role in DNA damage repair.

Interaction of cyclin D1 with DNA damage proteins

The first indications that cyclin D1 may play a direct role in DNA damage repair came from the observations functionally linking cyclin D1 with proteins involved in DNA repair. Richard Pestell's group showed that cyclin D1 tethered to chromatin can recruit RAD51, a protein that plays an essential role in homologous recombination process (17). Intriguingly, recruitment of RAD51 by cyclin D1 took place after DNA damage, but not in naive cells, suggesting a functional relevance of this interaction for DNA repair (17). The same group demonstrated a link between cyclin D1 and another DNA repair protein, BRCA1, by showing that cyclin D1 antagonizes BRCA1-mediated repression of estrogen receptor α transcriptional activity (18). The effect was mediated through an ability of cyclin D1 to compete with BRCA1 for ERα binding (18). A direct link between cyclin D1 and BRCA1 was provided by the observation that cyclin D1-CDK4 kinase phosphorylates BRCA1 on Ser-632; this event was shown to inhibit recruitment of BRCA1 to target promoters (19). BRCA1, as well as BRCA1 splice variant BRCA1-IRIS were shown to transcriptionally upregulate cyclin D1 (20, 21), while cyclin D1 was demonstrated to induce BRCA1 expression, through the pRB→E2F pathway (22).

A possible functional link between cyclin D1 and another DNA repair protein, BRCA2 was suggested by a study demonstrating that cyclin D1, BRCA2 as well as RAD51 physically interact with Sp1 transcription factor (23, 24).

Cyclin D1 was also shown to physically interact with two other proteins involved in DNA repair, PCNA (25, 26) and replication factor C (RFC, ref. 27). Both PCNA and RFC functionally interact with BRCA1 during repair of UV-induced DNA damage (28). Collectively, all these observations point to multiple functional links between cyclin D1 and DNA repair proteins suggesting that cyclin D1 may play a role in DNA damage repair.

Consistent with this view, several studies noted that elevated levels of cyclin D1 confer relative radiation-resistance to cancer cells (29–33). A correlation was noted between high levels of cyclin D1 and unfavorable response to radiotherapy (34, 35). Conversely, knock-down of cyclin D1 sensitized cancer cells to radiation or to DNA damaging agent cisplatin (33, 36). Moreover, antisense-mediated downregulation of cyclin D1 expression increased sensitivity of zebrafish embryos to radiation (37).

It should be noted, however, that many studies reached the opposite conclusion. For example, Coco-Martin et al. (38) demonstrated that ectopic overexpression of cyclin D1 sensitized cancer cells to irradiation, by rendering cells more susceptible to radiation-induced apoptosis. Similar conclusion was reached by Trent et al. (39) who proposed that the cyclin D1-driven enhancement of radiation sensitivity is CDK-independent, and it is mediated, at least in part, through the ability of cyclin D1 to transcriptionally induce expression of a heat shock protein HSPB8. According to some reports, cyclin D1 overexpression was shown to correlate with increased radiosensitivity and with favorable response to radio- or chemotherapy in squamous cell carcinoma of head and neck (40), oral squamous cell carcinoma (41) and in early-stage larynx cancer (42). It is possible that these conflicting observations reflect different types of tumors studied. Alternatively, these findings may illustrate a dual role for cyclin D1 in DNA damage response. Thus, following an acute DNA damage cyclin D1 levels need to be lowered to trigger cell cycle arrest, while the remaining pool of cyclin D1 may play an important, positive function in promoting DNA damage repair. Hence, strong overexpression of cyclin D1 to levels which cannot be efficiently reduced following DNA damage would compromise cell cycle checkpoints, and would impair cell survival, while elevated levels of cyclin D1 after DNA damage-induced cell cycle arrest might augment DNA repair. Likewise, a complete knock-down of cyclin D1 might compromise DNA repair, by depleting a protein that is needed for DNA repair.

A direct role of cyclin D1 in homologous recombination-based DNA repair

In a recent study (43) we utilized immunoaffinity purification of cyclin D1-containing complexes, followed by high-throughput shotgun mass spectrometry, to decipher the identity of cyclin D1-interacting proteins “cyclin D1-interactome” in several human cancer cell lines. Among cyclin D1-interactors we observed a strong enrichment for proteins belonging to “DNA damage repair” category.

One of these proteins was RAD51, an essential DNA recombinase which mediates homologous recombination-based DNA repair (44). An interaction between endogenous RAD51 and cyclin D1 was verified by immunoprecipitation-western blotting in a wide panel of human cancer cell lines, indicating that the interaction is not cell type-specific. Importantly, the interaction is strongly enhanced following irradiation of cells, suggesting that DNA damage might induce posttranslational modification of cyclin D1 and/or RAD51, which then stabilizes cyclin D1-RAD51 binding (43).

Surprisingly, we detected cyclin D1 at the sites of double stranded DNA breaks where it co-localized with RAD51, suggesting that cyclin D1 may directly participate in the repair of damaged DNA. We also observed that RAD51 recruitment to DNA damage sites was significantly reduced in cells depleted of cyclin D1. Moreover, knock-down of cyclin D1 reduced the rate of homologous recombination-mediated DNA repair (43). These findings are consistent with and fully support the observations of Li et al. (17) who demonstrated that cyclin D1, when targeted to chromatin, can recruit RAD51 following DNA damage. Collectively these findings suggest a model in which cyclin D1 localizes to double stranded DNA breaks following DNA damage, and helps to recruit RAD51 (Figure 1B).

How is then cyclin D1 recruited to broken DNA? Among cyclin D1-interacting proteins, detected in our screen, we observed BRCA2. Like RAD51, BRCA2 is also recruited to DNA damage sites. BRCA2 localization at the DNA damage foci represents a crucial step for homologous recombination, and it occurs prior to the recruitment of RAD51 (45). BRCA2 displaces the single stranded DNA (ssDNA) binding protein RPA from single stranded regions generated by end-resection, and facilitates loading of RAD51 onto ssDNA (45). We observed that depletion of cyclin D1 had no effect on BRCA2 recruitment to DNA damage

foci. However, knock-down of BRCA2 decreased loading of cyclin D1, suggesting that BRCA2 is responsible for recruiting cyclin D1 to DNA damage sites. Consistent with the observed association between BRCA2 and cyclin D1 in cancer cells, we found that purified recombinant cyclin D1 binds to purified BRCA2 fragments, in an *in vitro* binding assay.

We propose that cyclin D1 is recruited to double stranded DNA breaks via BRCA2. Cyclin D1 then helps either to recruit RAD51, or to stabilize RAD51 on the repair foci, thereby contributing to the homologous recombination process (Figure 1B).

Of note, we showed that the function of cyclin D1 in DNA repair is independent of its role in cell cycle progression. All our DNA repair analyses were performed in pRB-negative cancer cells, which do not require cyclin D1 for proliferation (46, 47). Moreover, the DNA repair role of cyclin D1 is independent of cyclin D1's ability to activate CDK4 and CDK6.

Possible implications for cancer treatment

Inhibition of cyclin D-associated kinase activity is currently being entertained as an attractive strategy for treatment of several cancer types (48), and inhibitors of cyclin D-CDK4 and D-CDK6 kinases are currently in clinical trials. Demonstration that cyclin D1 plays a CDK-independent function in DNA damage repair suggests that cyclin D1 protein (rather than cyclin D1-associated kinase) might represent a more effective anti-cancer target, as inhibition of cyclin D1 is expected to impair both cell proliferation, as well as DNA repair.

Another implication from our study is the function of cyclin D1 in pRB-negative cancer cells. It is very well documented that cancer cells that lost pRB no longer require D-cyclins for proliferation (46, 47). Consequently, depletion of cyclin D1, or inhibition of CDK4/6 kinase activity has no impact on proliferation of pRB-negative cancer cells. However, our study raises a possibility that targeting cyclin D1 might have a therapeutic value also in pRB-negative tumors, where it is expected to decrease the efficiency of DNA damage repair.

Unresolved issues

A direct role for cyclin D1 in DNA damage repair was detected in a screen which utilized human cancer cells. It remains to be seen whether cyclin D1 plays role in DNA repair also in normal cells. Consistent with this possibility, we observed that immortalized mouse embryonic fibroblasts lacking all three D-cyclins are more susceptible to radiation-induced DNA damage as compared to their wild-type counterparts.

Another unresolved issue is whether other D-type cyclins (D2 and D3) play a similar role in DNA repair. It will be interesting to determine whether these proteins also interact with RAD51 and with BRCA2, and are recruited to DNA damage sites.

A more fundamental question is whether D-cyclins, or other components of mammalian core cell cycle machinery affect the choice of DNA repair pathway. Mammalian cells can repair double stranded DNA breaks through error-prone non-homologous end joining (NHEJ), or through relatively faithful homologous recombination. The identity of molecules/signaling pathways that determine the choice of DNA repair pathway is not fully understood. Several reports showed that *bona fide* cell cycle CDKs can phosphorylate DNA repair proteins, and may determine the choice of DNA repair pathway. In budding yeast, it was shown that homologous recombination is controlled at an early step called DNA-end resection, by an activity of Cdc28 (a yeast homolog of mammalian CDKs) (49). Cdc28 phosphorylates Sae2 and a nuclease Dna2, key proteins in DNA-resection, thereby allowing progression of the DNA end-resection process, which represents a prerequisite for homologous recombination

(50, 51). Conversely, inhibition of yeast CDK activity during DNA damage causes yeast to employ NHEJ to repair DNA (51). A similar regulation was observed in human cells (52). However, the processes governing DNA repair pathways is mammalian cells seem to be more complex. For instance, cyclin A-CDK2 (and possibly cyclin B-CDK1) kinase was reported to block interaction between the C-terminus of BRCA2 and RAD51, and likely to inhibit homologous recombination, by phosphorylating Ser 3291 on BRCA2 (53). It is clear that the cell cycle and DNA repair machinery intersect at several points, and more work is needed to fully understand the functional interplay between these pathways.

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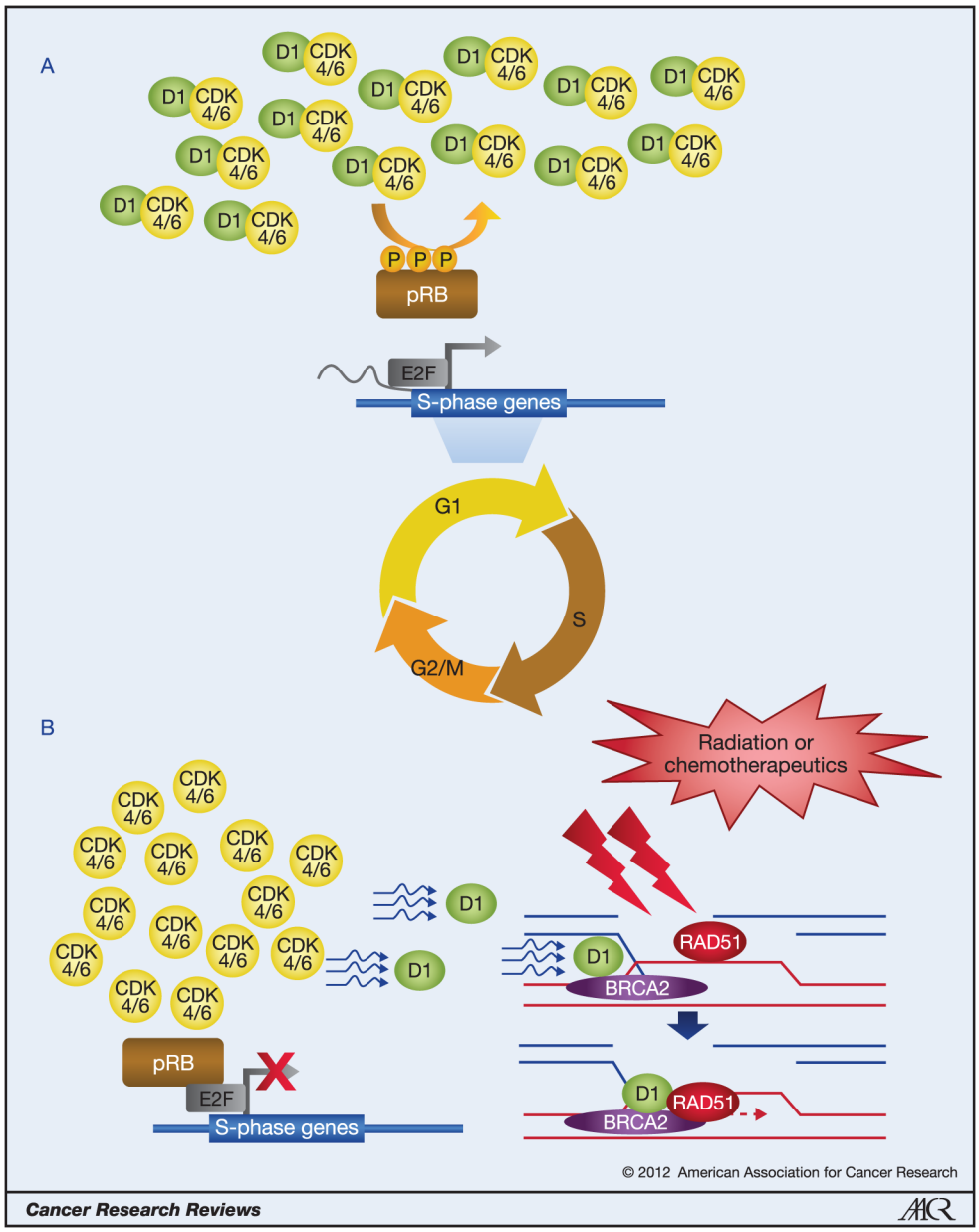

Figure 1. A model summarizing cyclin D1 function in cell proliferation and in DNA repair

A, During normal cell cycle progression, cyclin D1 (D1) forms complexes with CDK4 or CDK6 to regulate G1 phase progression by phosphorylating pRB, p107 and p130 proteins. pRB phosphorylation releases E2F transcription factors, and allows E2F-dependent transcription of S-phase genes.

B, After DNA damage caused by radiation or by chemotherapeutic agents, cyclin D1 protein levels are significantly reduced, and CDK4 kinase activity is diminished. Hypophosphorylated pRB inhibits E2F's ability to transactivate genes, thereby contributing to cell cycle arrest. Part of the remaining pool of cyclin D1 protein is recruited to DNA damage sites, in a BRCA2-dependent manner, to facilitate RAD51 localization or to stabilize RAD51 at the DNA damage sites, thereby assisting the homologous recombination-mediated DNA repair. Note that it is unclear whether CDK4 or CDK6 are recruited to DNA damage sites along with cyclin D1.