รายงานวิจัยฉบับสมบูรณ์

โครงการ

"บทบาทของไซคลิน ดี 1ในกระบวนการซ่อมแซมดีเอ็นเอแบบโฮโมโลกัสรีคอมบิเนชั่น"

โดย

ดร.ศิวนนท์ จิรวัฒโนทัย คณะแพทย์ศาสตร์ศิริราชพยาบาล

เดือนกรกฎาคม 2558

สัญญาเลขที่ RSA5580018

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยมหิดล

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. และมหาวิทยาลัยไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

โครงการวิจัยนี้สามารถสำเร็จลุล่วงไปได้ด้วยดี โดยได้รับความร่วมมือจากหน่วยงานและบุคคลหลายฝ่ายด้วยกันโดยเฉพาะอย่างยิ่ง ต้องขอขอบพระคุณต่อ สำนักงานกองทุนสนับสนุนการวิจัย (The Thailand Research Fund, TRF)

ขอขอบคุณภาควิชาเภสัชวิทยา คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล และผู้ร่วมงานทุกท่านที่ให้ความร่วมมือและสนับสนุนการดำเนินโครงการวิจัย

ดร.ศิวนนท์ จิรวัฒโนทัย

กรกฎาคม 2558

บทคัดย่อ

รหัสโครงการ : RSA5580018

ชื่อโครงการ : บทบาทของไซคลิน ดี 1 ในกระบวนการซ่อมแซมดีเอ็นเอแบบโฮโมโลกัสรีคอมบิเนชั่น

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ไซคลิน ดี 1 (cyclin D1) เป็นโปรตีนที่ควบคุมวัฏจักรของเซลล์ ซึ่งเป็นหนึ่งในโปรตีนที่เป็นสาเหตุในการ เกิดโรคมะเร็งในมนุษย์ และกระบวนการที่ โปรตีน ไซคลิน ดี 1 กระตุ้นก่อให้เกิดมะเร็ง ก็ยังไม่ชัดเจนนัก จากการวิจัยที่ผ่านมาพบว่า โปรตีน ไซคลิน ดี 1 มีบทบาทใหม่ ในการซ่อมแซมดีเอนเอ ซึ่งบทบาทใหม่นี้ มีผลให้เซลล์มะเร็งสามารถอยู่รอดได้ไม่ถูกกำจัดไป นอกจากนี้ โปรตีน ไซคลิน ดี 1 ยังมีส่วนช่วย ในกระบวนการซ่อมแซมดีเอนเอแบบhomologouse recombination (โฮโมโลกัสรีคอมไบเนชั่น) โดยช่วยทำให้ โปรตีน แรด 51 (RAD51) (ซึ่งเป็นโปรตีนที่มีส่วนช่วยในการซ่อมแซมดีเอนเอ) ไปยังตำแหน่งที่ดีเอนเอถูกทำลาย ดังนั้น โปรตีน ไซคลิน ดี 1 จึงเป็นหนึ่งในโปรตีนสำคัญ ที่จะเป็นเป้าหมายในการรักษาโรคมะเร็ง ในการวิจัยในครั้งนี้ ต้องการศึกษากลไกของโปรตีน ไซคลิน ดี 1 ที่ทำงานในกระบวนการซ่อมแซม ดีเอนเอแบบ โฮโมโลกัสรีคอมไบเนชั่น โดยเน้นที่ปฏิกิริยาของโปรตีน ไซคลิน ดี 1 และ โปรตีนบีอาซีเอ 2 (BRCA2) ในภาวะการควบคุมวัฏจักรของเซลล์ และการซ่อมแซมดีเอนเอแบบโฮโมโลกัสรีคอมไบเนชั่น ในโครงการนี้เราได้แสดงให้เห็นว่า ไซคลิน ดี 1 สามารถจับกับ C-terminus ของบีอาซีเอ 2 โดยใช้ส่วนอมิโนแอสิด 20-90 และการจับนั้นเพิ่มขึนหลังจากมีการทำลายของ ดีเอ็นเอ ที่น่าสนใจคือไซคลิน ดี 1กับ ซีดีเค 4 (CDK4) มิได้ฟอสโฟริเลต บีอาซีเอ 2ที่ Ser3291 โดยตรงแต่ในทางตรงข้ามกลับขวางไม่ให้ไซคลิน เอ (cyclin A) และ พาทเนอร์ซีดีเค 2 (CDK2) เข้ามาจับและฟอสโฟริเลท บีอาซีเอ 2 ที่ตำแหน่งดังกล่าวได้ จึงเป็นการช่วยให้แรด 51 เข้าจับกับ บีอาซีเอ 2 ได้โดยตรง ดังนั้นโครงการนี้แสดงให้เห็นว่าระดับและการแข่งขันของไซคลิน ต่างๆในเซลล์มีผลต่อการซ่อมดีเอ็นเอโดยปฏิกิริยานี้เกิดท่ส่วน C-terminus ของบีอาซีเอ 2

คำหลัก : ไซคลิน ดี **1;** ซีดีเค **2;** แรด **51;** บีอาซีเอ 2; Ser3291; โฮโมโลกัสรีคอมไบเนชั่น

Abstract

Project Code : RSA5580018

Project Title : Role of Cyclin D1 in Homologous Recombination (HR) DNA repair

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Project Period : 3 years

Cyclin D1 is cell cycle regulatory protein. It is also known as a cancer causing protein in human. A mechanism by which cyclin D1 promotes cancer formation is still unclear. Recently, we have established that cyclin D1 plays novel function in DNA repair and that this new function contributes to cancer cell survival. We have also shown that cyclin D1 promotes the homologous recombination (HR) DNA repair by facilitating a recruitment of RAD51 to DNA damage foci. To underline cyclin D1 as a potential therapeutic target for human cancers, we propose to study a detail mechanism by which cyclin D1 cooperates with the HR DNA repair machineries, emphasizing on an interaction between cyclin D1 and the Breast Cancer susceptibility protein BRCA2. 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

Project title: "Role of Cyclin D1 in Homologous Recombination (HR) DNA repair"

Introduction to the research problem and its significance

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 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, and 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. This ability of BRCA2 to gather 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.

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 event 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.

Objectives

To study role(s) of cyclin D1 in HR-based DNA repair, especially the interaction between cyclin D1 and the Breast cancer2 susceptibility protein, BRCA2, and a significance of the interaction in DNA repair and cell cycle control

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 the 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).

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

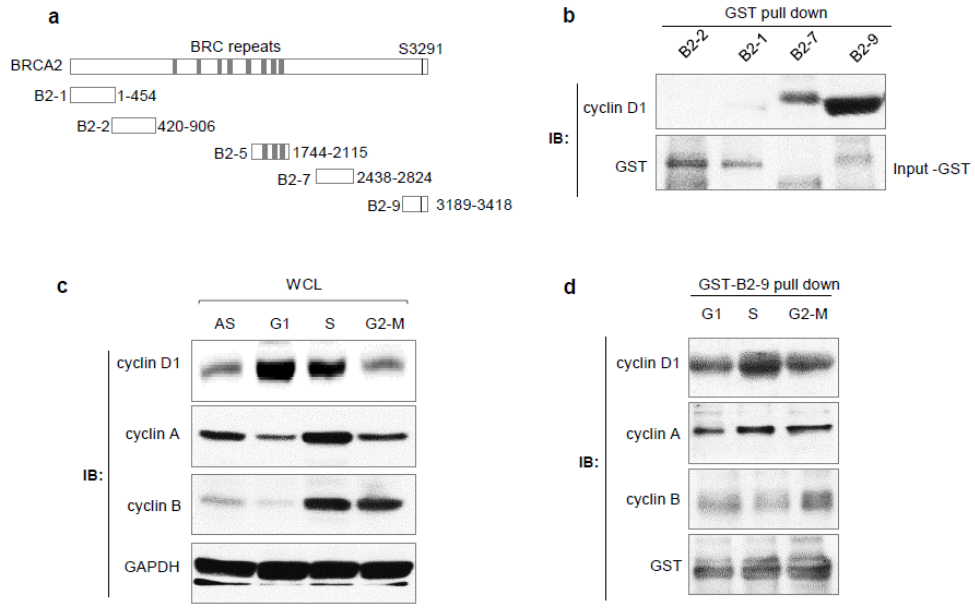

Figure 1. Cyclin D1 interacts with the C-terminus of BRCA2. a) Diagram depicting GST-BRCA2 fragments designated as B2-1, B2-2, B2-5, B2-7, B2-9 (39). The numbers adjacent to each fragment indicate the BRCA2 amino acids spanned by the fragments. Grey lines, BRC repeats; black line at the C-terminus indicates 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.

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 to 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.

Figure 2. Cyclin D1 suppresses BRCA2 Ser3291 phosphorylation

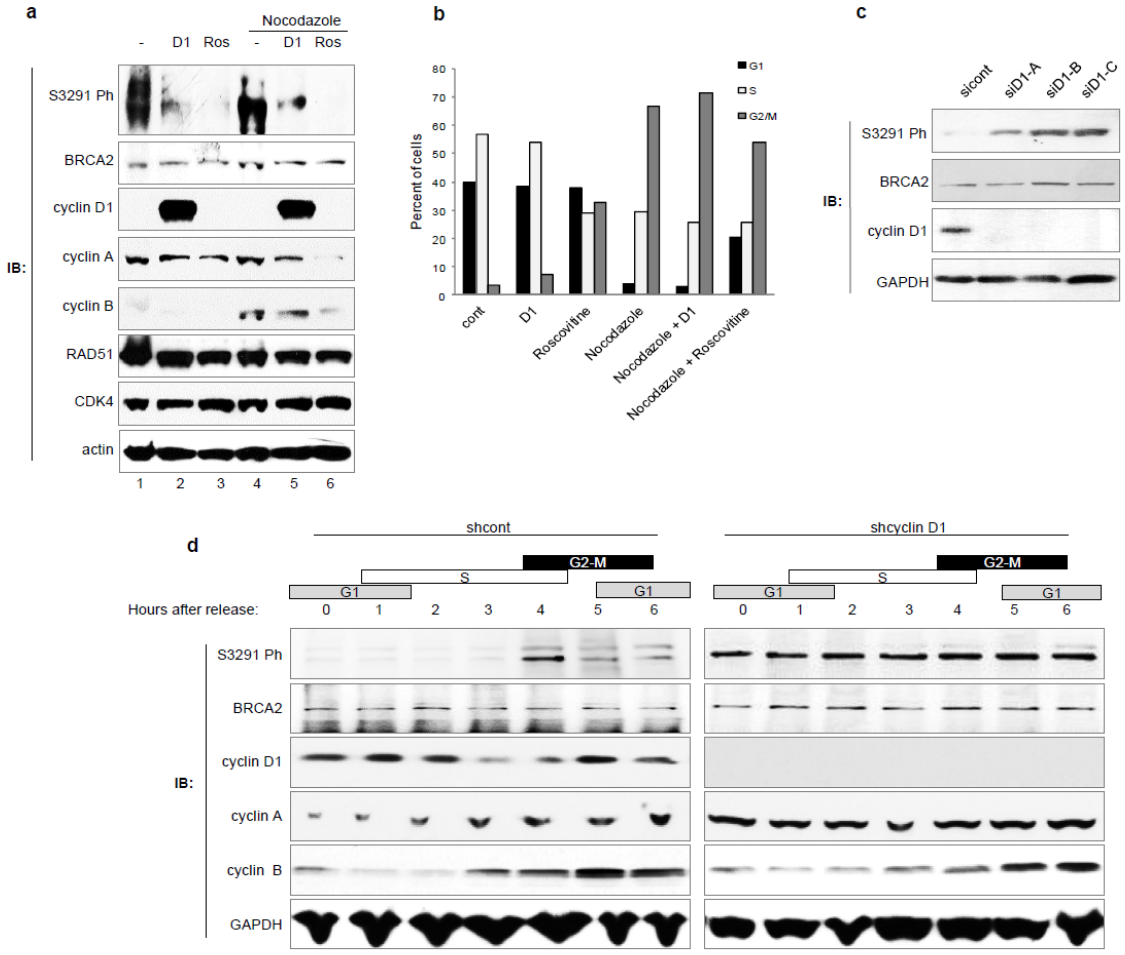

Figure 2. Cyclin D1 suppresses BRCA2 Ser3291 phosphorylation (a) Immunoblot (IB) analyses of phospho-Ser3291 BRCA2 (S3291 Ph) in HeLa cells, HeLa cells ectopically expressing cyclin D1 (D1), and HeLa cells treated with roscovitine (Ros). Lanes 1–3 contained lysates prepared from asynchronous cells, while 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.

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).

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

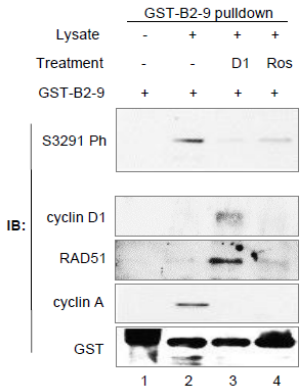

Figure 3. Cyclin D1 expression inhibits binding of cyclin A to the C-terminus of BRCA2 and promotes RAD51 binding. Immunoblot analyses of proteins co-precipitated with B2-9 under different conditions. Lane 1, B2-9 incubated with binding buffer alone; lane 2, B2-9 incubated in HeLa cell lysates. Lane 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.

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 that were composed of purified HA-tagged-cyclin D1 and GST-B2-9.

Compared with 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.

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

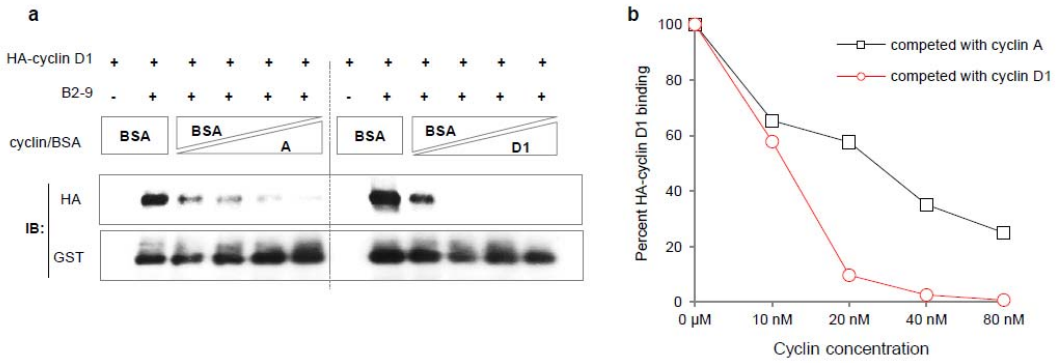

Figure 4. Competition between cyclin D1 and cyclin A for binding to the C-terminus of BRCA2. a) C-terminal fragment of BRCA2 (B2-9) was pre-mixed with purified HA–cyclin D1. Increasing amounts (0nM, 10nM, 20nM, 40nM, and 80nM) of either purified cyclin D1 or cyclin A were added to the reaction. Amounts 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).

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-CDK2/1dependent 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 re-localizes to the regulatory region C-terminus of BRCA2, thus, blocking Ser3291 phosphorylation by CDK2/1.

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 event mediated by other cyclins.

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

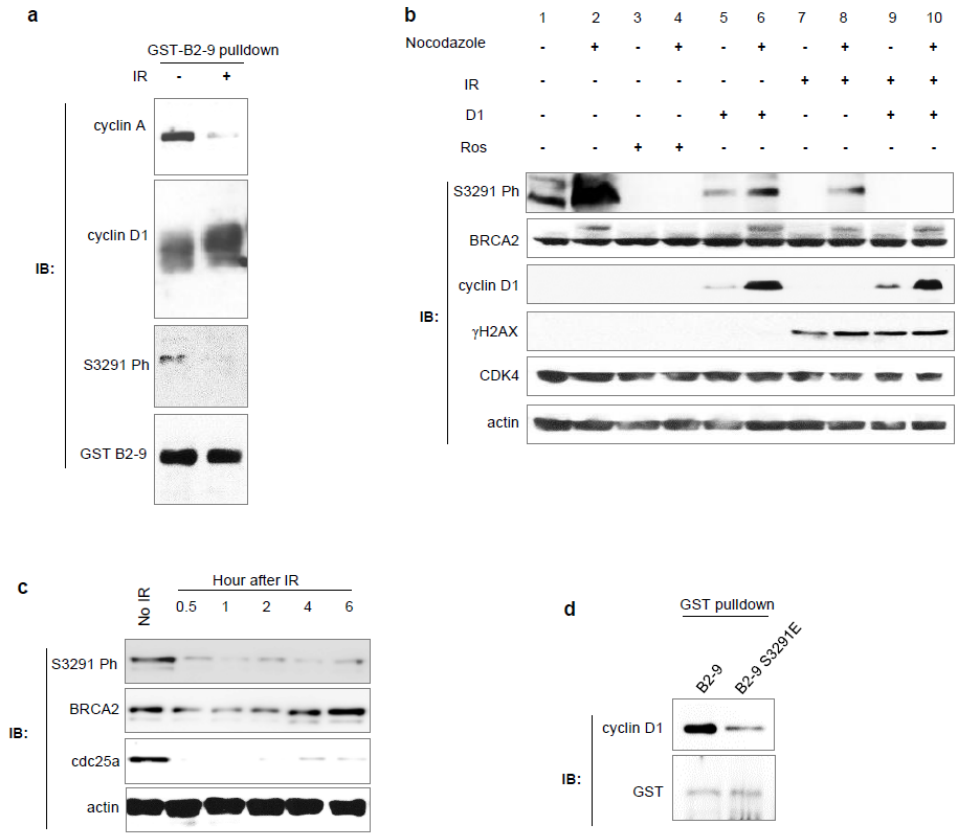

Figure 5. Cyclin D1 cooperates with DNA damage to inhibit BRCA2 phosphorylation at Ser3291. a) Co-precipitation of cyclin A and cyclin D1 at 0.5 hr after 5 Gy IR treatment. B2-9 was incubated with HeLa cell lysates prepared from cells with (+) or without IR treatment (-). Co-precipitated proteins were 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, γH2AX 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 andthe 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.

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 Δ1-19 that lacks amino acids 1–19 at the N-terminus of cyclin D1, and cyclin D1 Δ1–90 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 Δ1-19 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 Δ1–90 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 Δ1–90 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 (46-48), which is defective in phosphorylation-mediated nuclear export and subsequent proteolysis, 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.

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

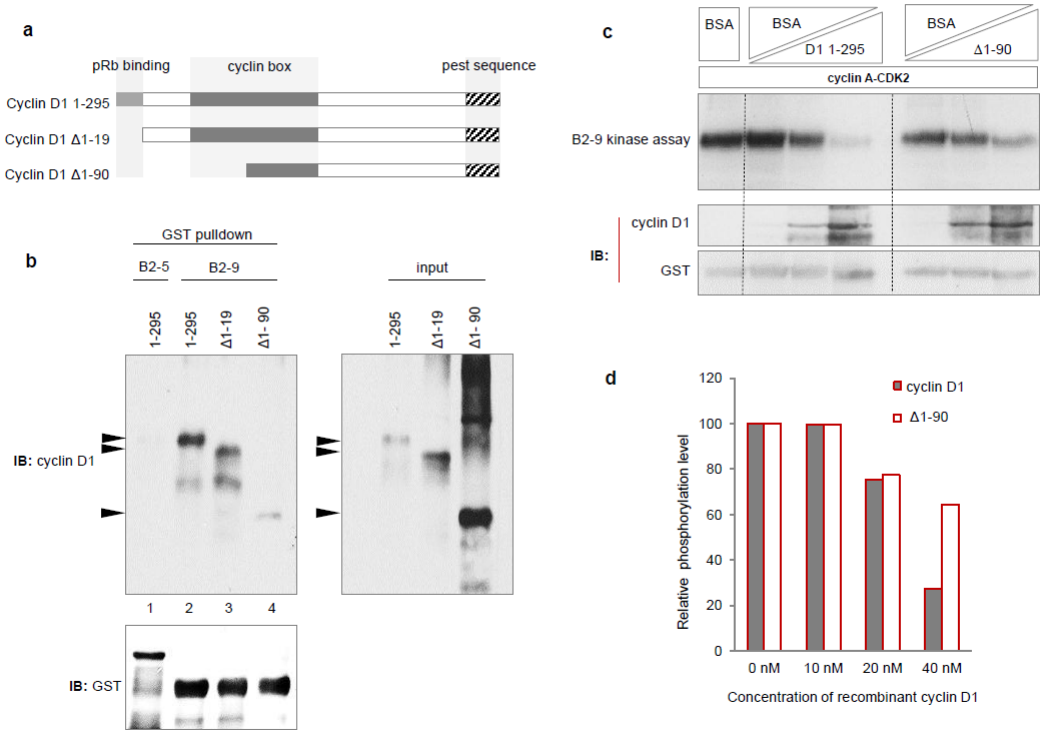

Figure 6. Amino acids 20–90 of cyclin D1 are required for BRCA2 C-terminus binding. a) Schematic diagrams of full-length (cyclin D1 1–295) and truncated mutants (Δ1–19 and Δ1–90). Light grey color highlights indicate known functional domains of cyclin D1, such as pRB binding domain, cyclin box, and pest 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 32P 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

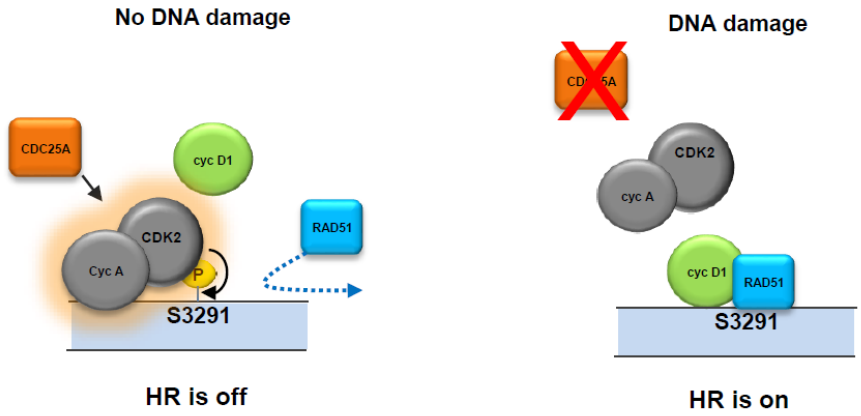

Figure 7. Prevention of BRCA2 Ser3291 phosphorylation by cyclin D1. When HR-repair is not required, i.e. no DNA damage, RAD51 recruitment to BRCA2 C-terminus is precluded by CDK2/1-dependent phosphorylation at BRCA2 Ser3291(left). However, when HR repair is required, cyclin A-CDK2-mediated phosphorylation 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).

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, in 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. On the other hand, the extended period of cyclin D1 overexpression was shown to cause genome instability (51, 52). One speculation is that, the elevated level of cyclin D1 can disturb the cyclins D1/A ratio in the cells, therefore, interfere 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 these agents (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.

For supplementary figures, please see in the APPENDIX

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 µg of each GST fusion protein was incubated overnight at 4°C in 1 mg of lysate. GST-BRCA2 fragments were pulled down using 20 µl 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 Gamma-^{32}P 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 μl 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 D1Δ1-90, 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, γH2AX antibodies (abcam, Cambridge, MA, USA), anti-β actin AKR-002, GAPDH AKR-001antibodies (Sigma-Aldrich). Anti-phospho-Ser3291 BRCA2 antibody was described previously (23).

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Output จากโครงการวิจัยที่ได้รับทุนจากสกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

1.1 ผลงานในโครงการที่ได้ตีพิมพ์และส่งสกว.แล้ว

The cyclin D1-CDK4 oncogenic interactome enables identification of potential novel oncogenes and clinical prognosis, Jirawatnotai S, Sharma S, Michowski W, Suktitipat B, Geng Y, Quackenbush J, Elias JE, Gygi SP, Wang YE, and Sicinski P, *Cell Cycle*, 2014;13(18):2889-900. doi: 10.4161/15384101.2014.946850. Impact factor 5.006

1.2 ผลงานที่กำลังอยู่ระหว่างกระบวนการการส่งเข้าสู่การพิจารณาเพื่อตีพิมพ์

Title: Cyclin D1 promotes RAD51 recruitment to the C-terminus domain of BRCA2

Under review in *Oncogene*

2. การนำผลงานวิจัยไปใช้ประโยชน์
   - เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)
     Establishing a research group in Thailand with high-impact research
   - เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

   Students in the project:

   Ms. Chonvara Chalermrujinanant, M.S. student

   Ms. Phatthamon Lapanuwat. Ph.D. student

3. อื่นๆ

3.1 ผลงานอื่น ๆ เช่น การไปเสนอผลงาน การได้รับเชิญไปเป็นวิทยากร

1. งานวิจัยนี้ถูกนำเสนอในงานประชุม The 7 th Asian Oceania Human Proteome Organnization (AOHUPO) Congress and the 9th International Symposium of Protein Society of Thailand 6-8th August, 2014, Miracle Grant, Bangkok, Thailand

2. งานวิจัยนี้ถูกนำเสนอในงานประชุม The Pharmacological and Therapeutic Society of Thailand-The 37th Congress on Pharmacology of Thailand "Genomic Medicine and Novel Cancer Therapy: Challenges and Opportunities" 28-30th May, 2015, Sunee Grant Hotel, Ubonratchathani Thailand

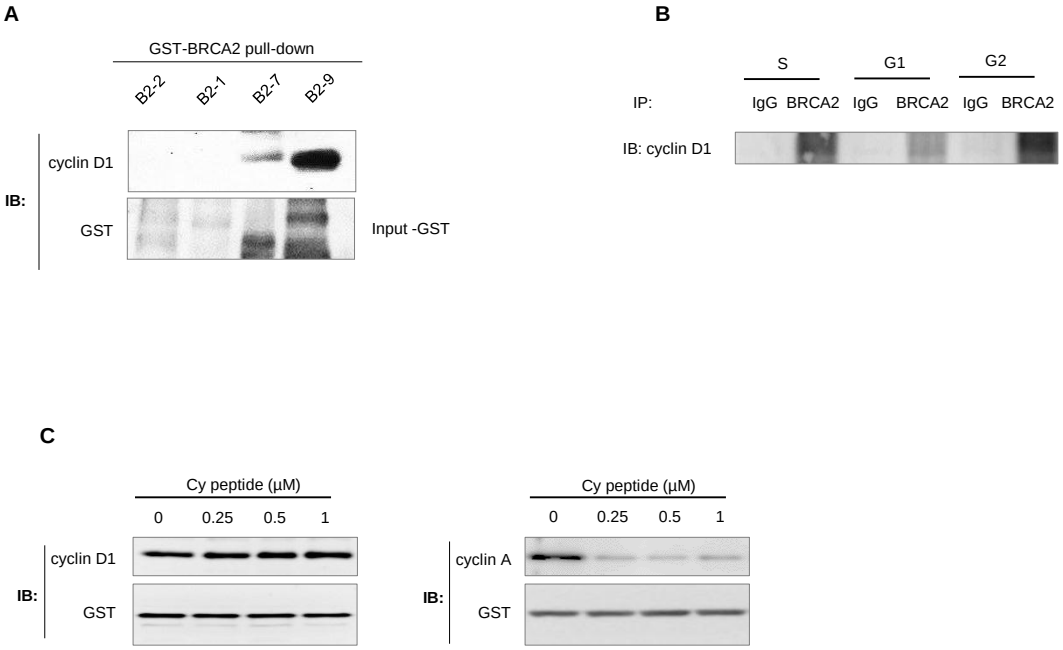
3.2 การเชื่อมโยงทางวิชาการกับนักวิชาการอื่นๆ ทั้งในและต่างประเทศ

มีความร่วมมือและแลกเปลี่ยนสารเคมีกับกลุ่มของ

1. Dr. Fumiko Esashi, Oxford University
2. Dr. Peter Sicinski, Harvard University
3. Dr. Wojicieck Michowski, Harvard University
4. Dr. Yaoyu Wang, Center for Computational Cancer Biology, Dana-Farber Cancer Institute
5. John Quackenbush, Center for Computational Cancer Biology, Dana-Farber Cancer Institute
6. Dr. David Livingston, Harvard University
7. Dr. Bhoom Suktitipat, Siriraj Hospital

APPENDIX I

Figure S1. Cyclin D1 interacts with C-terminal domain of BRCA2

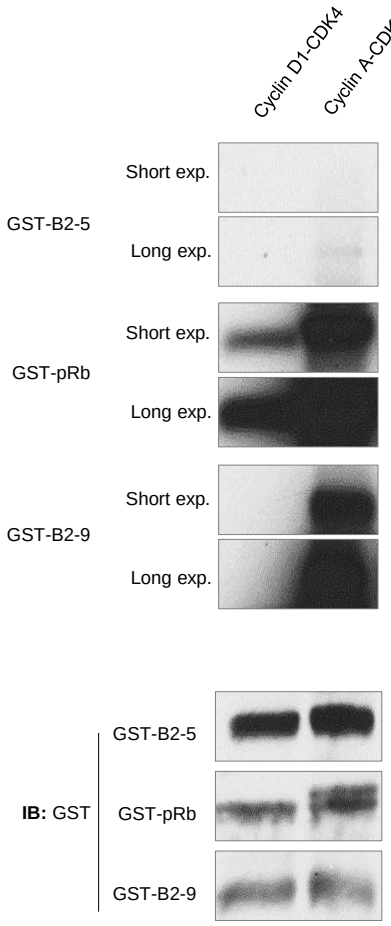


A. Interactions between GST-BRCA2 fragments and cyclin D1. B2-1, B2-7, and B2-9 were incubated with lysate prepared from asynchronous MCF7 cells. Endogenous proteins co-precipitated with the GST-BRCA2 fragments were analyzed by immunoblotting (IB) using indicated antibodies. GST immunoblot shows input GST-BRCA2 fragments.

B. BRCA2 interacts with cyclin D1 endogenously. Immunoprecipitations (IP) using anti-BRCA2 antibody were performed in HeLa lysates synchronized in G1, S, and G2 phase of cell cycle. Co-precipitated cyclin D1 was detected by IB using an anti-cyclin D1 antibody. Isotype control IgG (IgG) was used as a control for IP.

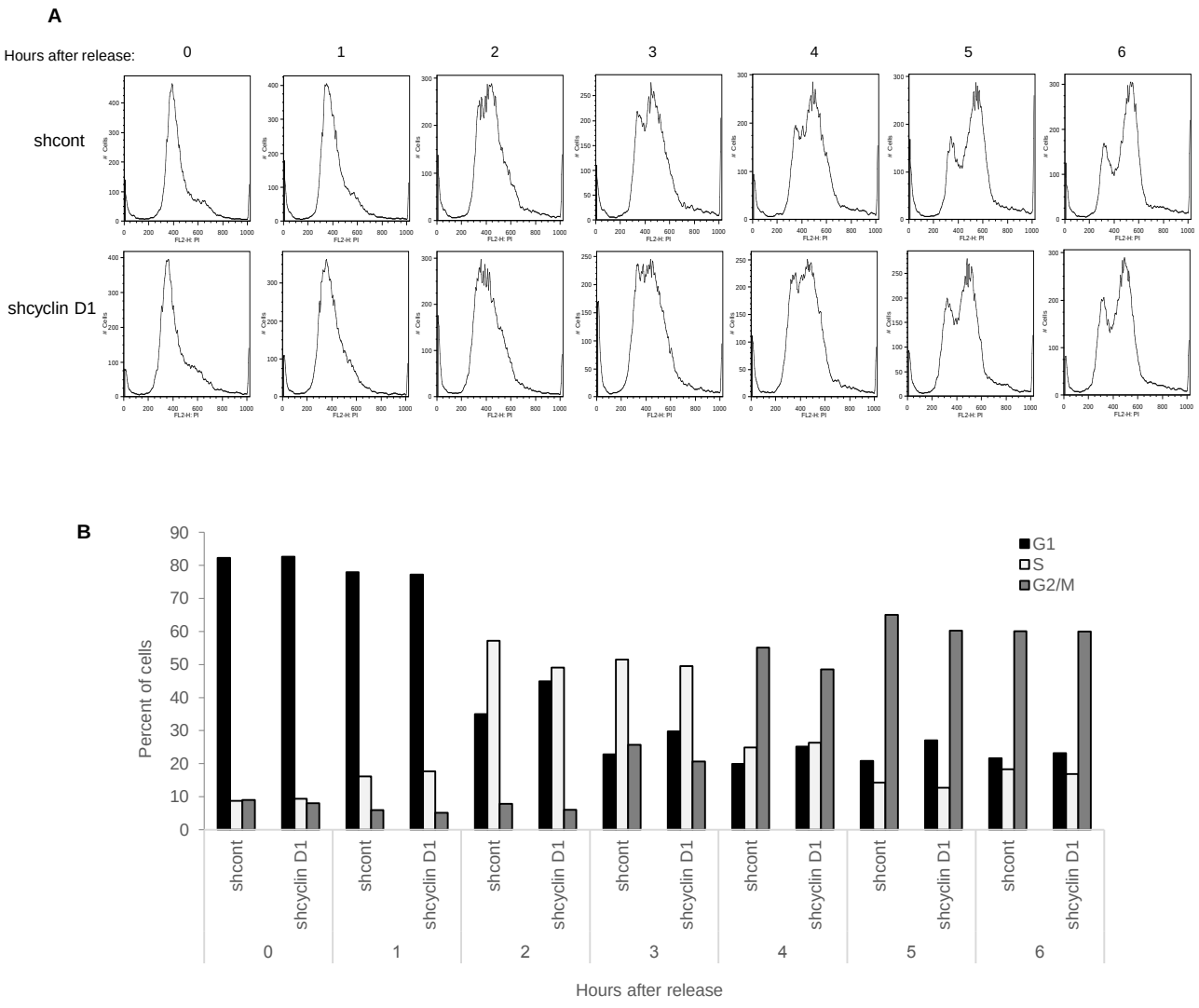
C. Independence of cyclin D1-B2-9 interaction on the cyclin recognition Cy site. *In vitro* binding reaction was performed by incubation of 10 ng of purified cyclin D1 (GST tag has been cut out), and 10 ng of GST-B29. The reactions were supplemented with a Cy peptide inhibitor as indicated. Cy peptide did not inhibit cyclin D1-B2-9 interaction (left panel). In a parallel experiment, cyclin D1 was replaced by purified cyclin A (right panel). Cy peptide interfered cyclin A-B2-9 interaction.

Figure S2. C-terminus of BRCA2 is a substrate for cyclin A-CDK2, but not for cyclin D1-CDK4



In vitro CDK kinase assays. CDK4-cyclin D1 and CDK2-cyclin A kinase assays were performed using B2-5, B2-9, and pRB as substrates. Amounts of each substrates were verified by immunoblotting (IB) using anti-GST antibody. Long exp; long exposure, short exp; short exposure.

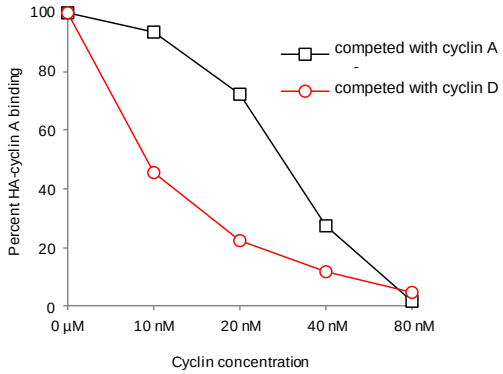
Figure S3. Cyclin D1 depletion by shRNA did not disturb cell cycle distribution of a pRb-negative cervical carcinoma cell line HeLa



A. Cell cycle profiles of HeLa cells expressing non-target control shRNA (shcont), or cyclin D1-specific shRNA (shcyclin D1) from Figure 2d. The pRB-inactivated cervical carcinoma HeLa cells were synchronized in late G1 with the double-thymidine blocks, then released into the cell cycle. Cell were harvested every hour (as indicated), and DNA contents were determined by propidium iodide staining and analyzed by FACS. Profiles of cyclin D1-depleted cells, and control cells were comparable

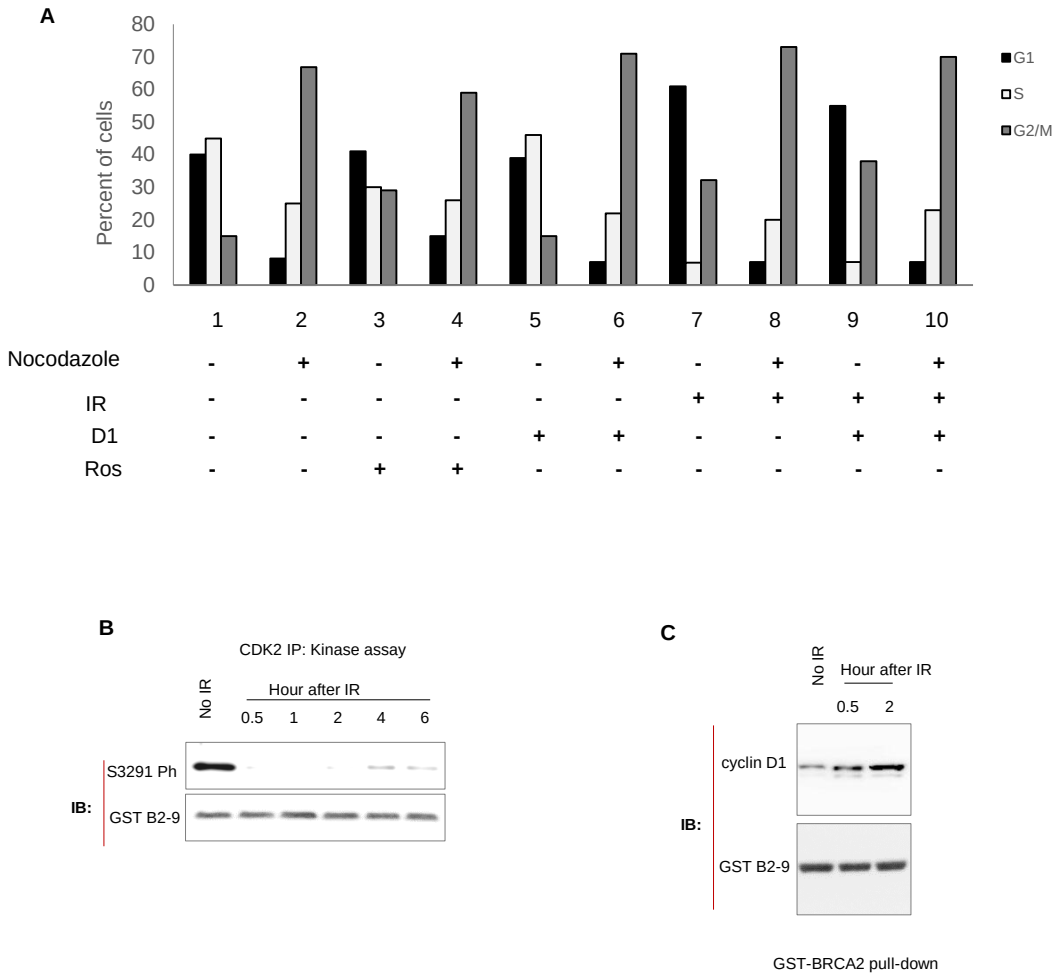
B. Bar graphs representation of percentages of cells from S3A, and Figure 2d in particular cell cycle phases

Figure S4. Cyclin D1 outcompetes cyclin A for BRCA2 C-terminus binding



Percentages of HA-cyclin A bound to B2-9 in the presence of purified cyclin D1 or cyclin A.

Figure S5. Cell cycle profiles of HeLa cells under various treatments

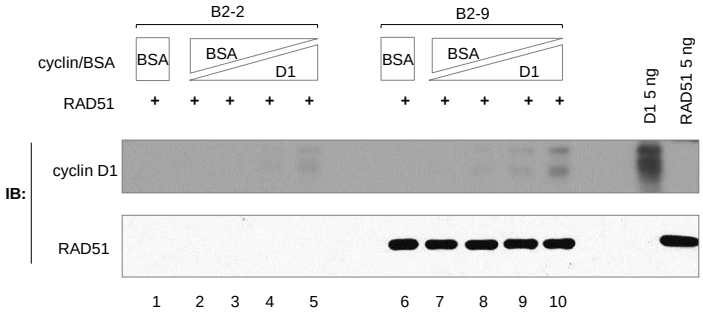


A. Cell cycle distribution of HeLa cells from Figure 5b. Cells were stained with propidium iodide and analyzed by FACS. Shown are percentages of cells in particular cell cycle phases.

B. CDK2-kinase activities after IR treatment. CDK2-cyclin complex was immunoprecipitated using a CDK2-specific antibody, and kinase activities on GST-B2-9 fragment at various time points were detected by Ser3291 Ph antibody. GST immunoblot indicated GST-B2-9 input.

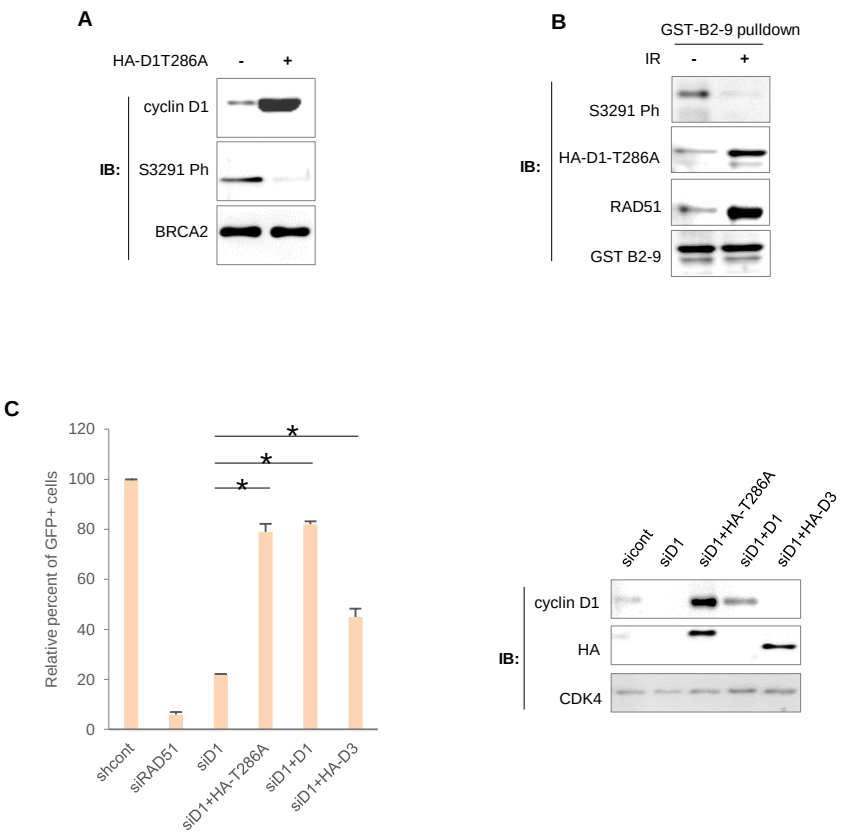
C. Cyclin D1-B2-9 interaction increased as early as 0.5 hr after DNA damage. GST-B2-9 was incubated in lysates from cell treated with 5 Gy IR at 0.5, 2 hrs, or no IR treatment. Cyclin D1 pulled down by B2-9 was detected using cyclin D1 immunoblot. GST immunoblot was used to determine input B2-9.

Figure S6. Purified cyclin D1 does not directly facilitate RAD51 binding to B2-9



In vitro binding of RAD51 and B2-9 in a presence of increasing amounts of cyclin D1. B2-2 was used as a negative control. Cyclin D1 or RAD51 that bound to B2-9 were analyzed by anti-cyclin D1 or anti-RAD51 antibody. Increasing level of cyclin D1 did not increase RAD51 interaction to B2-9. D1 5 ng and RAD51 5 ng were used to show inputs.

Figure S7. Phosphodegron cyclin D1 T286A binds to B2-9 and activates HR repair

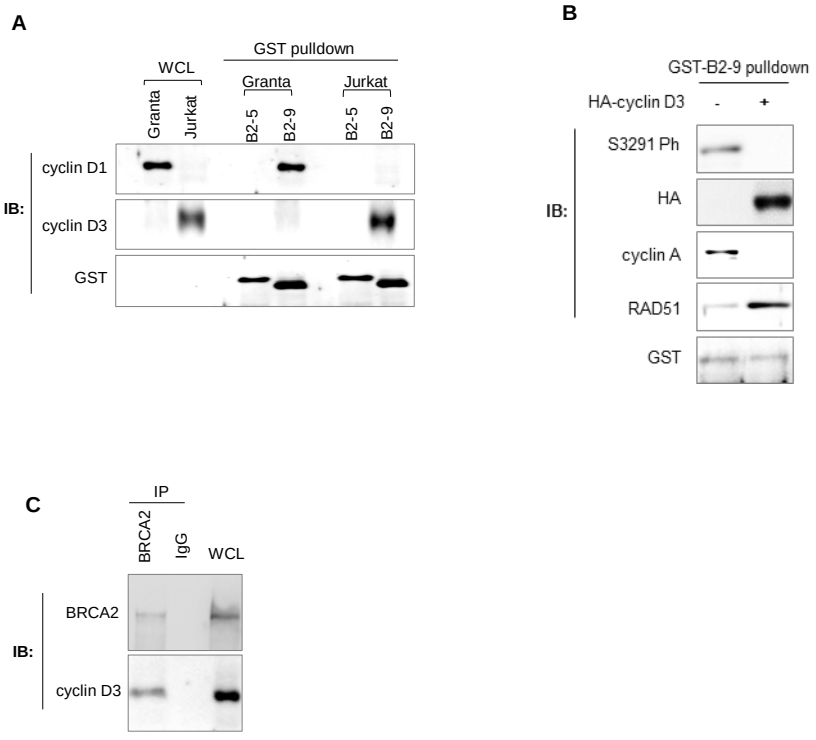


A. Expression of cyclin D1 T286A suppressed BRCA2 S3291 phosphorylation. pcDNA cyclin D1-HA T286A was transfected into HeLa cells. Levels of cyclin D1, S3291 Ph, and BRCA2 were analyzed by immunoblottings

B. Cyclin D1 T286A interacted with the C-terminus of BRCA2. GST-B2-9 was used to pull down cyclin D1 T286A, and RAD51 from cell lysate. The bindings of cyclin D1 T286A and RAD51 to B2-9 were enhanced in lysate prepared from cells treated with 5 Gy of IR. Level of phosphorylation on GST-B2-9 Ser3291 was analyzed by immunoblotting using the phospho-specific antibody

C. Homologous recombination assay (left panel) in HEK293 cells expressing an siD1-A resistant version of cyclin D1, HA-cyclin D1 T286A, and HA-cyclin D3 transfected with cyclin D1 siRNA-A (siD1 +D1, siD1 + HA-T286A, and siD1+HA-D3, respectively). Shown is percentages of GFP-positive cells, relative to cells transfected with control siRNA. Efficiency of homologous recombination in RAD51-depleted cells (siRAD51) is shown as reference. Right panel shows immunoblots of cyclin D1, HA, indicated the expression levels of the proteins. CDK4 immunoblot was used as loading control. Bars represent means of three independent experiments. HEK293 was used because it expresses very low level of al three D-type cyclins (data not shown). Error bars indicate S.D., **; $p \leq 0.05$*

Figure S8. Cyclin D3 interacts with B2-9 and suppress B2-9 Ser3291 phosphorylation



A. Interactions between GST-BRCA2 fragments and cyclin D3. GST-B2-9 was incubated with lysate prepared from Granta 519 (cyclin D1 overexpressing cells), or Jurkat cells (cyclin D3 overexpressing cells). Endogenous proteins, co-precipitated with the GST-BRCA2 fragments were analyzed by immunoblottings (IB) using indicated antibodies. GST immunoblot shows input GST-BRCA2 fragments, WCL; whole cell lysate. GST-B2-5 was used as the non-binding control.

B. The bindings of cyclin D3, cyclin A and RAD51 to B2-9 were enhanced in lysate prepared from cells overexpressing cyclin D3. Level of phosphorylation on GST-B2-9 Ser3291 was also analyzed by immunoblotting using the phospho-specific antibody. GST immunoblot shows input GST-BRCA2 B2-9.

C. BRCA2 interacts with cyclin D3 endogenously. Immunoprecipitations (IP) using anti-BRCA2 antibody were performed in Jurkat lysate. Co-precipitated cyclin D3, and BRCA2 were detected by IB using an anti-cyclin D3, and BRCA2 antibodies as indicated. Isotype control IgG (IgG) was used as a control for IP.

APPENDIX II

Cell Cycle
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/kccy20

The cyclin D1-CDK4 oncogenic interactome enables identification of potential novel oncogenes and clinical prognosis

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Published online: 30 Oct 2014.

To cite this article: Siwanon Jirawatnotai, Samanta Sharma, Wojciech Michowski, Bhoom Suktitipat, Yan Geng, John Quackenbush, Joshua E Elias, Steven P Gygi, Yaoyu E Wang & Piotr Sicinski (2014) The cyclin D1-CDK4 oncogenic interactome enables identification of potential novel oncogenes and clinical prognosis, Cell Cycle, 13:18, 2889-2900, DOI: 10.4161/15384101.2014.946850

To link to this article: http://dx.doi.org/10.4161/15384101.2014.946850

Cell Cycle 13:18, 2889–2900; September 15, 2014; © 2014 Taylor & Francis Group, LLC

The cyclin D1-CDK4 oncogenic interactome enables identification of potential novel oncogenes and clinical prognosis

Siwanon Jirawatnotai[1,*], Samanta Sharma[2], Wojciech Michowski[2], Bhoom Suktitipat[3], Yan Geng[2], John Quackenbush[4], Joshua E Elias[5], Steven P Gygi[6], Yaoyu E Wang[4], and Piotr Sicinski[2,*]

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Keywords: breast cancer, CDK4, cyclin D1, interactome, oncogenes, oncogenic signature

Abbreviations: ACN, acetonitrile; AES, aggregate expression score; ATCC, American type culture collection; DMEM, Dulbecco's Modified Eagle's medium; FBS, fetal bovine serum; LC-MS/MS, liquid chromatography-tandem mass spectrometry; PPI, protein-protein interaction; RPMI, Roswell Park Memorial Institute medium; SCNA, somatic copy-number variation; sicont, control small interfering RNA; sicyclin D1, cyclin D1-specific small interfering RNA; siFKBP4, FKBP4-specific small interfering RNA; siFKBP5, FKBP5-specific small interfering RNA; siRNA, small interfering RNA; TCGA, the cancer genome atlas; WB, immunoblotting.

Overexpression of cyclin D1 and its catalytic partner, CDK4, is frequently seen in human cancers. We constructed cyclin D1 and CDK4 protein interaction network in a human breast cancer cell line MCF7, and identified novel CDK4 protein partners. Among CDK4 interactors we observed several proteins functioning in protein folding and in complex assembly. One of the novel partners of CDK4 is FKBP5, which we found to be required to maintain CDK4 levels in cancer cells. An integrative analysis of the extended cyclin D1 cancer interactome and somatic copy number alterations in human cancers identified BAIAPL21 as a potential novel human oncogene. We observed that in several human tumor types BAIAPL21 is expressed at higher levels as compared to normal tissue. Forced overexpression of BAIAPL21 augmented anchorage independent growth, increased colony formation by cancer cells and strongly enhanced the ability of cells to form tumors in vivo. Lastly, we derived an Aggregate Expression Score (AES), which quantifies the expression of all cyclin D1 interactors in a given tumor. We observed that AES has a prognostic value among patients with ER-positive breast cancers. These studies illustrate the utility of analyzing the interactomes of proteins involved in cancer to uncover potential oncogenes, or to allow better cancer prognosis.

Introduction

Cyclin D1 belongs to the core cell cycle machinery. Once induced, it binds and activates the cyclin-dependent kinases CDK4 and CDK6. Cyclin D-CDK4/6 holoenzymes phosphorylate proteins governing cell cycle progression, such as the retinoblastoma protein, pRB.[1] Overexpression of cyclin D1 is found in several human cancer types, including breast cancers[2] colon cancers,[4,5] squamous cell carcinomas,[6] multiple myelomas,[7] and mantle cell lymphomas.[8] Many of these cancers contain amplification or rearrangements within the cyclin D1 (*CCND1)* locus. Indeed, *CCND1* represents the second most frequently amplified gene across all human cancer types.[9] Targeted overexpression of cyclin D1 using transgenic mouse models led to formation of malignant lesions, thereby providing a direct evidence for the causative role of cyclin D1 overexpression in oncogenesis.[10-12] Moreover, the continued presence of cyclin D1 is required for maintenance of the malignant phenotype, as an acute ablation of cyclin D1 in breast cancer-bearing mice blocked cancer progression.[13] Collectively, all these findings point to cyclin D1 as an attractive target for cancer therapy.[14] Cyclin D1 also plays roles beyond cell cycle progression,[15] and it is highly expressed in non-proliferating, senescent cells.[16,17]

Developments of high-throughput platforms and bioinformatic analyses have helped to reveal novel information about disease-causing proteins. Recently, we constructed a protein-protein interaction (PPI) network of cyclin D1 (cyclin D1 interactome) from 5 different human cancer cell lines representing mammary, squamous cell and colorectal carcinomas and mantle cell lymphoma.[18] This oncogenic cyclin D1 network was composed of 132 proteins. Gene ontology analyses revealed that in cancer cells cyclin D1 interacts with proteins regulating cell cycle and

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Submitted: 06/13/2014; Revised: 06/18/2014; Accepted: 06/19/2014
http://dx.doi.org/10.4161/15384101.2014.946850

proteins functioning in DNA repair pathways, both of which play roles in cancer formation.[19]

Because of a remarkable involvement of cyclin D1 overexpression in human cancers, we hypothesized that the cyclin D1 interactome may be enriched for cancer-causing proteins, and may allow identification of new oncogenes. We further hypothesized that the expression levels of cyclin D1 interactors (cyclin D1 interactome signature) may allow one to stratify cancer patients for prognostic reasons. To test these predictions, in this study we performed integrative analyses of cyclin D1 interactomes with the list of copy number alterations in human cancers, and with The Cancer Genome Atlas (TCGA)[20] breast cancer dataset. In addition, we constructed a joint PPI network of cyclin D1 and its kinase partner, the cyclin-dependent kinase 4 (CDK4), in a human breast cancer cell line and identified novel cyclin D1- and CDK4-interacting proteins.

Results

An oncogenic cyclin D1-CDK4 interactome in breast cancer cells

To determine the identity of cyclin D1 and CDK4 interactors in breast cancer cells, we expressed tandemly (Flag- and HA)-tagged versions of cyclin D1 and CDK4 in a human breast cancer cell line MCF7. We then used sequential immunoaffinity purifications with anti-Flag and anti-HA antibodies, followed by repeated rounds of liquid chromatography-tandem mass spectrometry (LC-MS/MS)[18] to determine the identity of cyclin D1[18] and CDK4 interacting proteins. Integration of the results allowed us to construct breast cancer cyclin D1-CDK4 oncogenic network (**Fig. 1A and B**; **Tables S1 and S2**). Surprisingly, we found very little overlap between protein partners of cyclin D1 and those of CDK4. The great majority of cyclin D1 interactors were not found in CDK4 immunoprecipitates and *vice versa* (**Tables S1 and S2**). The only protein detected both in cyclin D1 and CDK4 immunoprecipitates was a cell cycle inhibitor p18INK4C (*CDKN2C*) (**Fig. 1B**).

To verify the results of mass spectrometry analyses, cyclin D1 and CDK4 were immunoprecipitated from MCF7 cells, and immunoblotted with antibodies against various cyclin D1 and CDK4 interactors. We confirmed that cyclin D1-specific partners, including a novel interactor - zinc finger ZFP106 protein, were not found in anti-CDK4 immunoprecipitates (**Fig. 1C**). Conversely, CDC37, FKBP4, and FKBP5 proteins were confirmed as CDK4-specific interactors (**Fig. 1C**).

We next analyzed the biological function of cyclin D1 and CDK4 interactors with DAVID software.[21,22] We found that the majority of cyclin D1 partners such as CDK4, CDK2, p21 (*CDKN1A*), p27(*CDKN1B*) belonged to "cell cycle" category (**Fig. 1B**). In contrast, most of CDK4 interactors (67%) represented proteins involved in protein folding and complex assembly. Among CDK4 interactors, we observed several previously unknown partners, such as FKBP4, FKBP5, CAD, CCT2, CCT4, and PRDX6.

To better understand the stoichiometry of cyclin D1-CDK4 interaction, we performed immunodepletion experiments. First, we immunodepleted cyclin D1 from MCF7 lysates. We found that a nearly complete depletion of cyclin D1 eliminated only a small fraction of CDK4 (**Fig. 1D**, second lane). In contrast, when CDK4 was depleted from the lysates, the majority of cyclin D1 pool was also depleted (**Fig. 1D**, third lane). This indicates that in MCF7 cells, the great majority of cyclin D1 molecules interacts with CDK4 (the rest binds to other CDKs and to cyclin D1-specific partners, such as ZFP106, **Fig. 1B**). On the other hand, only a small portion of CDK4 molecules interacts with cyclin D1 and most of CDK4 molecules reside in protein complexes that are devoid of cyclin D1 (**Fig. 1C and E**). This cyclin D1-free pool of CDK4 interacts with proteins involved in protein folding and in complex assembly. We observed that proteins of the Hsp90 kinase chaperone complex, CDC37 and Hsp90, interacted with CDK4 outside of the cyclin D1-CDK4 complex (**Figs. 1B**, **2A and B**).[23-25] As shown in **Fig. 1C**, no interaction between CDC37 and cyclin D1 was observed in MCF7 cells (**Fig. 1C**).

FKBP5, a novel CDK4-binding protein, is required for full CDK4 expression and kinase activity

Our mass spectrometry analyses revealed that CDK4 interacts with several previously unknown partners representing chaperone proteins, such as members of the CCT complex, as well as with 2 members of FK506-binding family, FKBP4 and FKBP5 (**Figs. 1B and 2A**). The interaction of CDK4 with FKBP4 and FKBP5 was confirmed by immunoblotting (**Fig. 1C**). FKBPs represent a family of proteins that bind to immunosuppressive compounds such as FK506, rapamycin, and cyclosporin A. FKBPs are involved in several biochemical processes including protein folding, receptor signaling, and transcription.[26,27] We verified the interaction between CDK4 and FKBP5 in a panel of human cancer cell lines. FKBP5 co-precipitated with CDK4 in every cell line tested, suggesting that the interaction is ubiquitous (**Fig. 2C**). Since FKBP5 appeared to be one of the most abundant CDK4-binding proteins, as judged by our mass spectrometry analyses (**Fig 2A**; **Table S1**), we tested whether FKBP5 is required for CDK4 function and stability. Depletion of FKBP5 using 2 independent siRNAs significantly reduced the levels of CDK4 protein (**Fig. 2D**), and diminished CDK4 kinase activity (**Fig. 2E**). Depletion of another CDK4 interactor, FKBP4 led to only modest reduction of CDK4 levels (**Fig 2F**). Collectively, these findings suggest that FKBP5 acts as CDK4 chaperonin and plays a role in controlling CDK4 levels.

Previous reports indicated that CDC37 physically interacts with CDK4 and stabilizes CDK4 protein levels.[23] Given our observation that FKBP5 appeared to play a similar role, we investigated whether FKBP5 is a part of the CDK4-CDC37 complex. Using MCF7 cells expressing Flag-tagged CDK4, we immunoprecipitated CDK4 with anti-Flag antibody, eluted the complex from the beads using the Flag peptide, re-immunoprecipitated the complex with anti-FKBP5 (**Fig. 2G**, lane 5) or CDC37 (**Fig. 2G**, lane 6) antibodies, and immunoblotted with antibodies against FKBP5 and CDC37. We found that CDK4 interacts with FKBP5 or with CDC37, but it does not form a ternary CDK4-FKBP5-

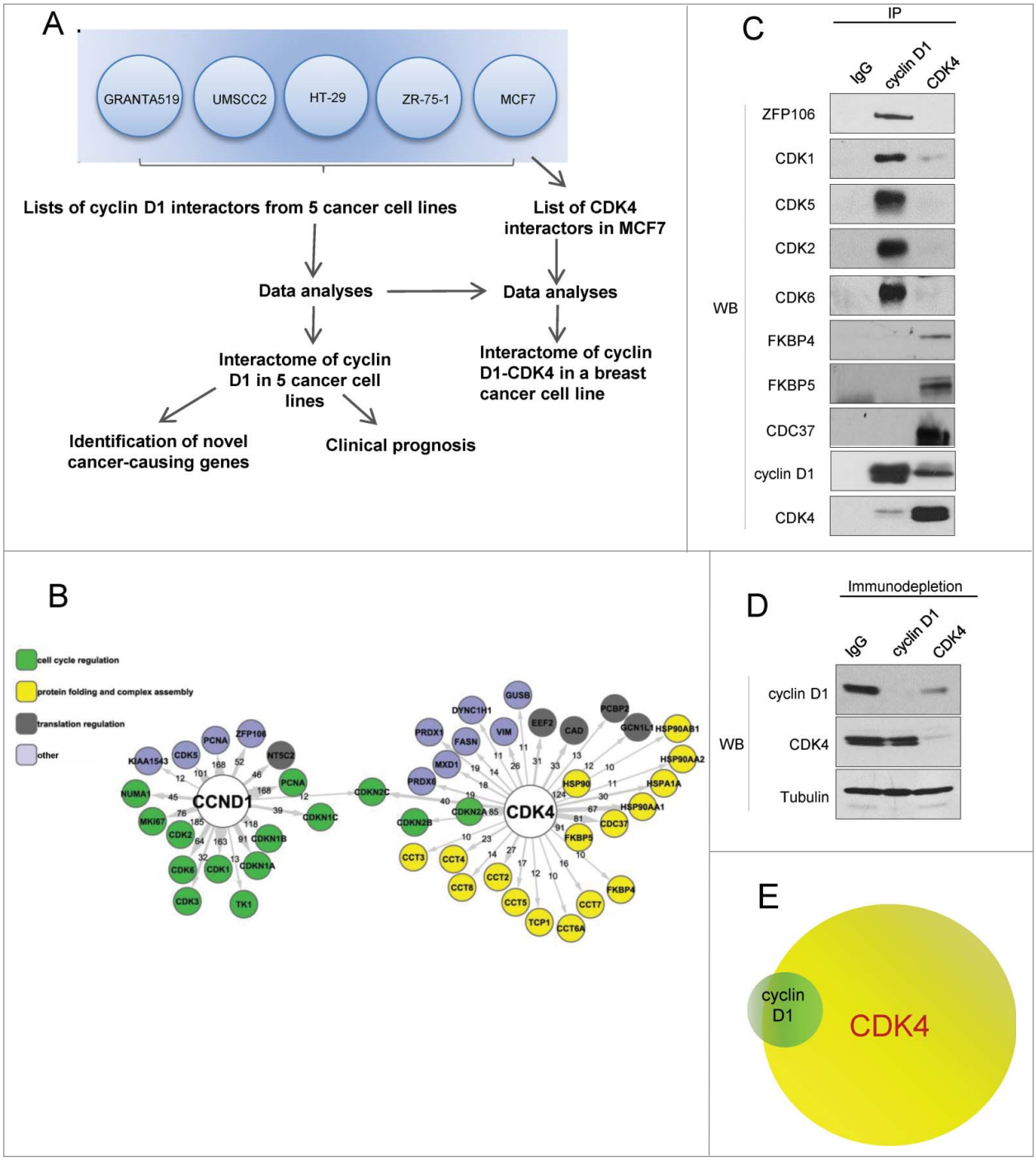

Figure 1. Oncogenic cyclin D1-CDK4 interactomes in human cancer cells (**A**) Flow chart of the interactome analyses. Cyclin D1 joint interactome was made from 5 human cancer cell lines and was already published.[18] CDK4 interactome was obtained by analyzing a breast cancer cell line MCF7. (**B**) The oncogenic cyclin D1-CDK4 interactome in MCF7 cells. Colors indicate biological processes implicated for each of the interactors. Interactors are labeled by their gene names. Lines indicate whether a protein was detected as cyclin D1 (CCND1) or CDK4-associated protein; numbers on the lines represent number of spectra for a given protein detected in mass spectrometry analyses. The interactome of cyclin D1 was previously published.[18] (**C**) Immunoprecipitation (IP) - immunoblot (WB) verification of cyclin D1 and CDK4 interacting proteins. Cell lysates were immunoprecipitated with either anti-cyclin D1 or anti CDK4 antibody, or isotype-matched mouse IgG (IgG). Immunoprecipitated products were resolved on SDS-PAGE gels and immunoblotted with the indicated antibodies. (**D**) Immunodepletion of cyclin D1 or CDK4. Lysates were depleted with anti-cyclin D1 or anti-CDK4 antibodies; isotype-matched IgG was used as a control. Supernatants were immunoblotted with the indicated antibodies. (**E**) Proposed stoichiometry of cyclin D1-CDK4 interaction in MCF7 cells. The majority of cyclin D1 molecules (green) interact with CDK4 (yellow), while only a small fraction of CDK4 forms complex with cyclin D1. Please note that the sizes of circles representing cyclin D1 and CDK4 are hypothetical, as we do not know the absolute number of cyclin D1 and CDK4 molecules per cell.

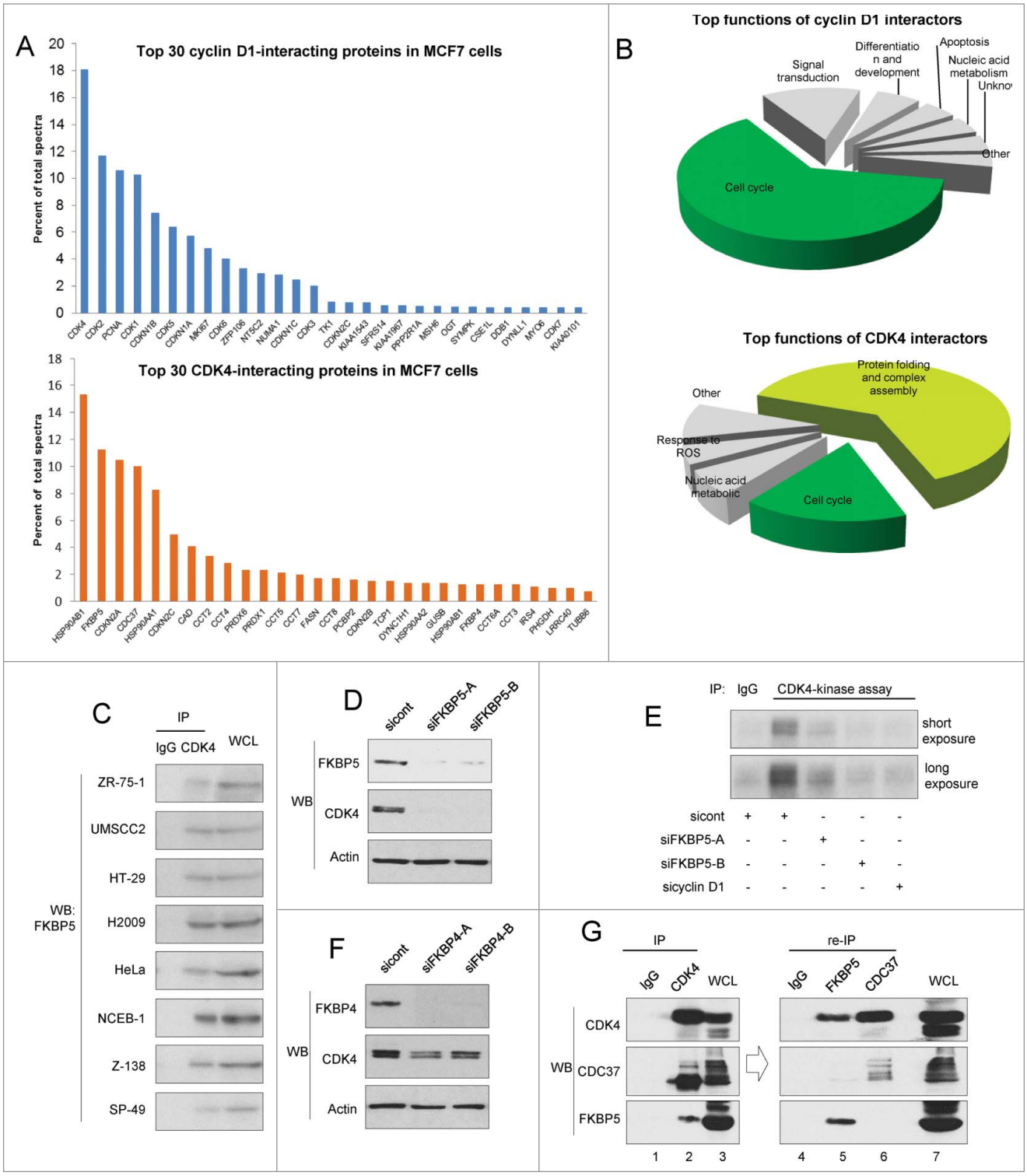

Figure 2. For figure legend, see page 2893.

CDC37 complex. These observations suggest that FKBP5 stabilizes CDK4 level, via a CDC37-independent mechanism.

Integrative analysis of cyclin D1 interactome and somatic copy number alterations in human cancers uncovers a potential oncogene, BAIAP2L1

Previously, we have identified cyclin D1 interactomes from 5 human cancer cell lines, MCF7, ZR-75–1, UMSCC2, HT-29, and Granta519.[18] Such interaction networks have been shown to represent a good source for uncovering biological functions of the participating proteins. One of the approaches, called "guilt-by-association" principle,[28] predicts a function of a novel protein based on known functions of its interactors. We applied a reverse logic to the cyclin D1 interactome. Given the fact that cyclin D1 represents a well-established oncogene, we asked whether cyclin D1 partners are enriched for proteins implicated in oncogenesis. To this end, we interrogated the cyclin D1 interactome for the presence of genes associated with tumorigenesis, as defined by Gene Census data (n = 513 genes) obtained from the COSMIC database.[29] Among cyclin D1 interactors we observed 12 proteins that have been causally implicated in cancer development (CDK6, FANCD2, CDKN2C, NUMA1, NONO, RB1, CDK4, XPO1, PPP2R1A, MSH6, BRCA2, and IKZF1). This number is significantly larger than that expected by a random chance ($p = 1.15X10^{-5}$, Fisher Exact test). These observations suggested to us that the cyclin D1 interactome may contain additional, currently unknown cancer-relevant proteins.

To identify these proteins, we intersected our interactome with a comprehensive list of genomic regions found to be frequently amplified or deleted in a large-scale analysis of over 3,000 of human cancers.[9] We searched for interactors whose genes map to commonly amplified or deleted regions in the human cancer genome. Genes encoding 6 cyclin D1 interactors: CDK4, CDK6, IKZF3, PHGDH, CCT2, and BAIAP2L1 map to peaks of commonly amplified chromosomal cancer regions (**Fig. 3A**, highlighted in red), whereas 4 cyclin D1 interactor genes: RB, TRIM28, ZNF324, and MKI67 are located within the peak deleted areas (**Fig. 3A**, highlighted in gray). These numbers indicated a significant enrichment for amplified and deleted genes within the cyclin D1 interactome ($p = 0.0015$, Fisher Exact Test) and suggested that some of these genes may encode previously unknown cancer-relevant proteins.

To test this notion, we focused on a novel cyclin D1 interacting protein with relatively unknown function, *BAIAP2L1* (BAI1-associated protein 2-like 1), a phosphorylation substrate for insulin receptor.[30] Since the gene encoding *BAIAP2L1* is amplified in human cancers (please see above), we hypothesized that this protein may have oncogenic properties. Prior evidence suggested that BAIAP2L1 may play a role in cancer formation. Thus, a fusion protein between a growth factor receptor FGFR3 and BAIAP2L1 was reported to be expressed in bladder cancer cells.[31] Moreover, BAIAP2L1 was also shown to play a role in cell migration.[32] We tested the impact of BAIAP2L1 on cell proliferation by ectopically expressing it in MCF7 cells. We observed that overexpression of BAIAP2L1 significantly increased colony forming ability of MCF7 cells (**Fig. 3B**). BAIAP2L1 overexpression also enhanced colony formation in another human cancer cell line, an osteosarcoma U2OS cells (**Fig. 3C**).

To test the impact of BAIAP2L1 expression on anchorage-independent growth, we overexpressed BAIAP2L1 in immortalized murine 3T3 cells, or in 3T3 cells engineered by us to express activated Ha-Ras. Ectopic expression of BAIAP2L1 endowed 3T3 cells with the ability to grow in soft agar, and enhanced anchorage-independent growth of Ras-expressing 3T3 cells (**Fig 3D**). BAIAP2L1 expression not only increased the total number of foci, but it also increased the number of large foci in Ha-Ras-transformed 3T3 cells (**Fig. 3D**). Surprisingly, immunoblot analyses revealed that ectopic expression of BAIAP2L1 significantly augmented the levels of Ha-Ras in Ras-transformed cells (**Fig 3E**).

We also tested the impact of BAIAP2L1-overexpression on the ability of cells to form tumors in immunocompromised mice. To this end, we injected BAIAP2L1-overexpressing 3T3 cells under the skin of nude mice. While none of the mice subcutaneously implanted with 3T3 cells developed any tumors after 8 weeks, 6 out of 7 (85.57%) mice injected with BAIAP2L1-expressing 3T3 cells formed tumors (**Fig. 3F**; **Table 1**). BAIAP2L1 appeared to be a stronger transformation inducer for the murine 3T3 cells than Ras. Thus, at 6 weeks post-inoculation, tumors triggered by BAIAP2L1 expression were significantly larger than those induced by Ras (**Fig. 3G**; **Table 1**).

Figure 2 (See opposite page). Analyses of cyclin D1 and CDK4 interactors in breast cancer cells (**A**) Top 30 cyclin D1 and CDK4 interactors detected by mass spectrometry analyses. (**B**) Top biological processes of cyclin D1 and CDK4 interactors. (**C**) Physical interaction between CDK4 and FKBP5 in various human cancer cell lines. CDK4 was immunoprecipitated in the indicated cell lines, and immunoblots were probed with an anti-FKBP5 antibody. Isotype-matched IgG was used as a control. (**D**) The impact of FKBP5 depletion on CDK4 levels. MCF7 cells were transfected with 2 independent anti-FKBP5 siRNAs (siFKBP5-A and siFKBP5-B), or with control siRNA (sicont), and the levels of FKBP5 and CDK4 were gauged by immunoblotting (WB) using the indicated antibodies. Actin was used as a loading control. (**E**) *In vitro* CDK4 kinase assays. MCF7 cells were transfected with siRNAs against FKBP5 (siFKBP5-A and siFKBP5-B), cyclin D1 (sicyclin D1) or with a control non-targeting siRNA (sicont). CDK4 was immunoprecipitated (IP) and used for *in vitro* kinase reactions with the retinoblastoma protein as a substrate. Immunoprecipitation with isotype-matched IgG (IgG) was used as a negative control. Short exposure: 30 min, long exposure: 3 hrs. (**F**) The impact of FKBP4 depletion on CDK4 levels. The experiment was performed as in panel D, except that 2 independent anti-FKBP4 siRNAs were used (siFKBP4-A and siFKBP4-B). (**G**) CDK4 immunoprecipitation followed by re-immunoprecipitation of either FKBP5 or CDC37. Flag-tagged CDK4 was immunoprecipitated (IP) from MCF7 cells using an anti-Flag antibody; complexes were then eluted with 3XFlag peptide. Ten percent of the eluent was resolved on an SDS-PAGE gel and immunoblotted with the indicated antibodies (left panel). The remaining eluent was split into 3 equal parts. One part was subjected to re-immunoprecipitation with anti-FKBP5 antibody (lane 5), the second with anti-CDC37 antibody (lane 6), the third with IgG (for control, lane 4). Co-immuprecipitated proteins were then detected with the indicated antibodies (right panel). IgG was also used for control immunoprecipitation in lane 1. WCL; whole cell lysate (lanes 3 and 7).

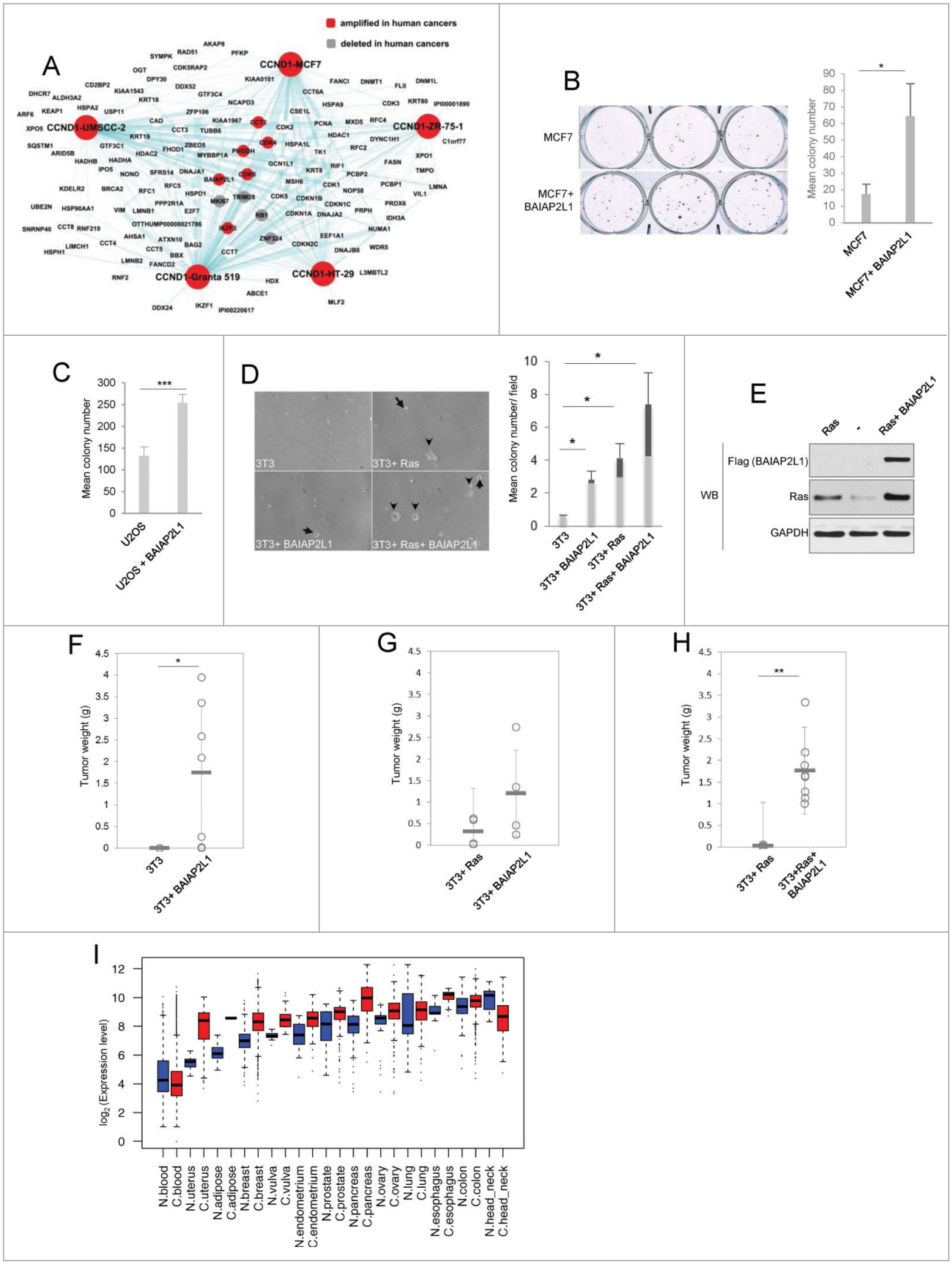

Figure 3. For figure legend, see page 2895.

Table 1. Tumor formation in nude mice

Cells	Number of mice with a tumor at endpoint	Percent of mice with a tumor at endpoint	Time to endpoint
3T3	0/7	0	N/A
3T3 + Ras	8/12	66.7	>6 weeks
3T3 + BAIAP2L1	6/7	85.7	4-8 weeks
3T3 + Ras + BAIAP2L1	8/8	100	4 weeks

Numbers and percentages of mice with tumors at the endpoints are indicated. Mice injected with 3T3 cells were observed for 8 weeks, and no tumors were detected. N/A, not applicable.

Moreover, cells expressing BAIAP2L1 formed tumors in a higher fraction of mice than cells expressing Ras (85.57% vs. 66%, **Table 1**). Co-expression of Ras and BAIAP2L1 in 3T3 cells synergistically enhanced the ability of cells to form tumors, and increased both tumor weight as well as the fraction of mice with tumors (**Fig. 3H**; **Table 1**). Tumors co-expressing Ras and BAIAP2L1 grew so rapidly, that the endpoint of the experiment had to be shortened to only 4 weeks (**Fig. 3H**; **Table 1**). These analyses indicate that *BAIAP2L1* has several properties of an oncogene.

We next compared the levels of BAIAP2L1 transcripts in various cancer types, versus in corresponding normal tissues using GENT (Gene Expression Across Normal and Tumor Tissue) database.[33] We were able to query BAIAP2L1 expression across 32 tumor types and in normal tissues from 21,434 samples (17,931 tumor samples and 3,503 normal tissue samples). Mean expression level of BAIAP2L1 in tumor samples was higher than that seen in normal tissues (303.80 ± 443.85 vs 235.93 ± 386.38 [mean± SD], $p = 1.6019 \times 10^{-17}$, log-rank test). Levels of BAIAP2L1 expression in tumor samples were then compared to the corresponding types of normal tissues using linear mixed model, which allows each type of tissue to have different baseline expression of BAIAP2L1. Among the 32 types of tumors and normal tissues, the cancer-normal matched samples were available for analyses of 25 tumor types (see Materials in Methods). In these 25 tumor types, BAIAP2L1 expression levels were 1. fold17- higher in tumor samples than in normal tissues (95%CI from 1.12 to 1.24, $p = 4.25\text{x}10^{-10}$). Thirteen tumor-normal matched types showed statistically significant differences in BAIAP2L1 expression levels between tumors vs. normal samples ($p < 0.002$) (**Fig. 3I**; **Table S3**). This list includes malignancies of blood, uterus, adipose tissue, breast, vulva, endometrium, prostate, pancreas, ovary, lung, esophagus, colon, and head and neck. In all pairs, except blood and head-neck cancers, BAIAP2L1 was expressed at higher levels in cancer samples than in normal counterparts (**Fig. 3I**, boxplots). These observations are consistent with a growth-promoting function for BAIAP2L1 in cancer cells, and support the notion that BAIAP2L1 likely represents a *bona fide* oncogene.

A gene signature generated from cyclin D1 interactome holds prognostic value for survival of breast cancer patients

Since the cyclin D1 interactome is enriched for potential cancer-relevant genes, we hypothesized that expression levels of cyclin D1 interactors (cyclin D1 interactome signature) might contain prognostic value for survival of cancer patients. To test this, we focused on estrogen receptor-positive breast cancers due to the availability of clinical data that utilizes large cohorts of patients, and given the well-established overexpression of cyclin D1 in this breast cancer subtype.[34] We interrogated the TCGA breast cancer data set and imputed the data to retain only

Figure 3 (See opposite page). Analyses of cyclin D1 interactome to identify novel cancer-relevant genes (**A**) Cyclin D1 interactome from 5 cancer cell lines (this interactome was already presented in Jirawatnotai et al.[18]) Nodes represent gene symbols of cyclin D1 interactors. Lines depict interactions found by our mass spectrometry analyses. Genes found to be amplified in human cancers[9] are highlighted in red, genes deleted in cancers[9] in gray. (**B**) MCF7 colony formation assay. MCF7 cells stably expressing BAIAP2L1 (MCF7 + BAIAP2L1) or cells transfected with an empty vector (MCF7) were seeded at a low density, and colonies were stained with crystal violet and enumerated after 3 weeks. Right panel shows mean colony numbers, error bars represent standard deviation (n = 3), *; $P < 0.05$. (**C**) U2OS colony formation assay. U2OS cells were engineered to stably express BAIAP2L1 (U2OS + BAIAP2L1), or were transfected with an empty vector (U2OS) and used for assays as in panel B. Bar graphs represent mean colony numbers, error bars standard deviation (n = 3); ***, $P < 0.005$. (**D**) Soft agar assay of murine 3T3 cells stably expressing BAIAP2L1 (3T3+ BAIAP2L1), Ras (3T3 + Ras), Ras + BAIAP2L1 (3T3 + Ras + BAIAP2L1), or transfected with an empty vector (3T3). Left panel: microscopic images of representative fields. Right panel: Bar graphs showing mean total colony numbers; dark gray bars show mean numbers of large colonies (200 cells or more). Error bars, standard deviation; *, $P < 0.05$ (**E**) Protein levels of Ras and BAIAP2L1 in Ras- and in Ras + BAIAP2L1 transduced 3T3 cells, determined by immunoblotting (WB). Anti-Flag antibody was used to detect ectopically expressed Flag-BAIAP2L1. (**F**) Weights of BAIAP2L1-induced tumors at 8 weeks post inoculation. Mice were injected subcutaneously with 3T3 cells (3T3) or with 3T3 cells stably expressing BAIAP2L1 (3T3 + BAIAP2L1). Each circle corresponds to a separate tumor, horizontal lines depict mean values; *, $P < 0.05$ (**G**) Weights of Ras- or BAIAP2L1-induced tumors at 6 weeks post inoculation. Mice were injected subcutaneously with 3T3 cells stably expressing activated Ras (3T3 + Ras) or BAIAP2L1 (3T3 + BAIAP2L1). Each circle corresponds to a separate tumor, horizontal lines depict mean values. (**H**) Weights of Ras- or Ras + BAIAP2L1-induced tumors at 4 weeks post inoculation. Mice were injected subcutaneously with 3T3 cells stably expressing activated Ras (3T3 + Ras) or Ras plus BAIAP2L1 (3T3 + Ras + BAIAP2L1). Each circle corresponds to a separate tumor, horizontal lines depict mean values; **, $P < 0.001$ (**I**) Boxplots of log-2 transformed expression levels of BAIAP2L1 transcripts in 13 tissue types with differential expression in cancer versus in normal tissue. Source of material is described on the x-axis: N- denotes normal and C- denotes cancer.

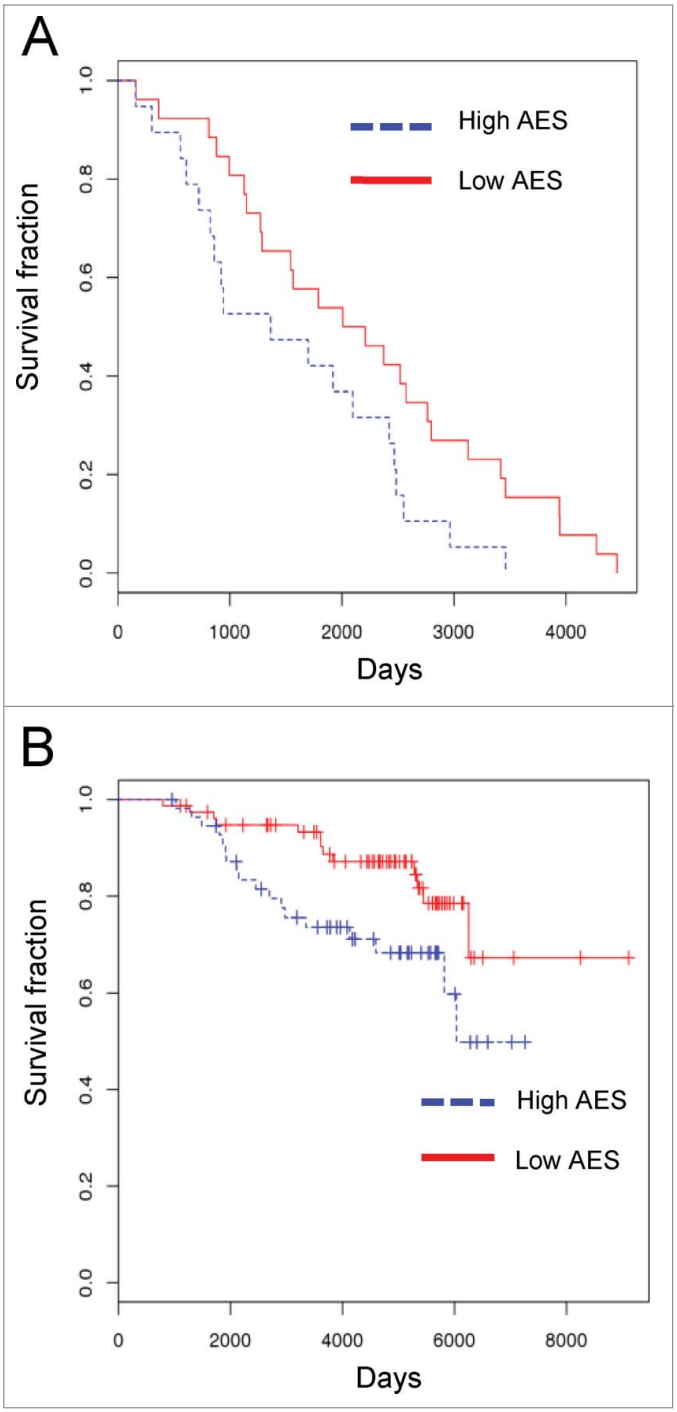

Figure 4. Prognostic value of cyclin D1 interactome-based signature (**A**) ER-positive breast cancers from TCGA data set were stratified by cyclin D1 interactome Aggregated Expression Score (AES). Red and blue lines indicate survival of patients with low and high AES scores, respectively. $p = 0.042$ (**B**) Patients with ER-positive breast cancers from TRANSBIG cohort were stratified and analyzed as in **Fig. A**. Red and blue linesindicate survival of patients with low and high AES scores, respectively. $p = 0.032$.

individuals with confirmed ER-positive mammary carcinomas and with known survival status (n = 45).

We first asked whether cyclin D1 expression correlates with patient survival. We observed no such correlation in our cohort (data not shown). Next, we used Aggregate Expression Score (AES) (as described in Materials and Methods), to assess the prognostic value of cyclin D1 interactome levels. Briefly, AES summarizes the overall expression level of the interactome in a given tumor, by summing up the number of genes expressed one standard deviation above (+1) and one standard deviation below (−1) of the mean expression levels seen across all cancer samples (please see Materials and Methods). We then stratified the patients into AES high and AES low groups to assess if interactome expression level correlates with survival. We found that high AES of cyclin D1 interactome correlated with poor survival rate in patients bearing ER-positive breast cancers ($p = 0.042$, log-rank test) (**Fig. 4A**). In contrast, the interactome of CDK4 had no predictive value (data not shown). To verify our observation in an independent cohort, we repeated the same analysis using the TRANSBIG data set,[35] which contains a large number of patients with ER-positive breast cancers (n = 134). Again, we observed that the cyclin D1 interactome AES had a predictive value for patients survival; with high AES corresponding to poor survival (KM-plot, $p = 0.032$, log-rank test) (**Fig. 4B**).

Discussion

Recently, we have generated an integrated oncogenic cyclin D1 interactome from several human cancer cell lines.[18] In the current study, we analyzed the cyclin D1 interactome in conjunction with other data sets, namely in the context of the CDK4 interactome, somatic copy-number alterations (SCNA), gene expression, and clinical data sets from human cancer patients to extract additional contextual biological information.

Our analyses of the CDK4 interactome revealed that a member of the FKBP family (FKBP5) represents a novel chaperone regulating CDK4 protein levels and kinase activity. Several studies postulated that FKBP5 may play a role in tumorigenesis. Thus, FKBP5 was shown to stimulate androgen-dependent transcriptional activation and to promote prostate cancer growth.[36] Ectopic overexpression of FKBP5 was demonstrated to increase radioresistance of melanoma cells.[37] However, other studies implicated FKBP5 as a negative regulator of cell growth, by inhibiting the AKT signaling pathway. Specifically, overexpression of FKBP5 was shown to decrease the activating AKT-Ser473 phosphorylation, whereas depletion of FKBP5 had an opposite effect.[38] We observed that FKBP5 functions to stabilize CDK4. We found that depletion of FKBP5 led to decreased CDK4 protein levels and decreased CDK4 kinase activity. Given the well-established role of CDK4 hyperactivation in tumorigenesis, it seems likely that FKBP5 promotes oncogenesis in part by stabilizing CDK4.

Analyses across several thousands of human tumors led to delineation of regions commonly amplified (or commonly deleted) in cancers.[9] However, these regions are usually large (median length of 1.8 Mb, range 0.5 kb-85 Mb), and hence they

contain a substantial number of genes.[9,39] One of the main challenges is to identify "driver" cancer causing genes that reside in the commonly amplified or deleted regions. We hypothesized that by overlaying copy number alteration data with cancer cell interactome of a well-established oncogene (cyclin D1), we might uncover novel cancer-causing genes. Indeed, we demonstrated that cyclin D1 interactome is enriched for known oncogenes and tumor suppressor genes. Moreover, we used the intersection of the interactome and copy number alteration analyses to identify BAIAP2L1 as a potential novel oncogene.

We found that *BAIAP2L1* has several characteristics of a *bona fide* oncogene. First, the *BAIAP2L1* gene is amplified in several cancer types.[9] Second, the gene is expressed at higher levels in cancer samples, as compared to normal, non-transformed counterparts. Third, ectopic overexpression of *BAIAP2L1* increased colony formation of MCF7 and U2OS cancer cells. Fourth, BAIAP2L1 overexpression was sufficient to trigger anchorage-independent growth of 3T3 cells, and endowed 3T3 cells with an ability to form tumors in nude mice. Further studies are needed to decipher the exact molecular mechanism of BAIAP2L1 oncogenic role. As stated above, we observed that BAIAP2L1 expression increased protein levels of the oncogenic Ras. Of note, it was recently reported that BAIAP2L1 can activate EGFR/ERK signaling pathway and promote cell proliferation of hepatocellular carcinoma.[40]

Our study examined the utility of using the interactome of an oncogene as a prognostic tool in cancer patients. We devised an AES scoring method to represent the interactome expression level in a given tumor, and then evaluated the prognostic value of AES to predict survival using the TCGA breast cancer cohort. The recentness of this cohort substantially limited the power of survival analysis, yet the score of cyclin D1 interactome correlated with survival of ER-positive breast cancer patients. In contrast, the levels of cyclin D1, on its own, had no any predictive value. This suggests that the interactome adds substantial prognostic information. We further confirmed our findings by analyzing the TRANSBIG cohort[35] as a testing set. To the best of our knowledge, this is the first instance of using aggregate gene expression scoring of an oncogenic interactome to construct a prognostic signature. While there are several published multigene signatures, mostly derived from expression profiling,[41-45] our method yielded comparable statistically significant prognostic value despite its simplicity.[35]

Since the cyclin D1 interactome AES was built from 4 diverse types of human tumors, it may have a predictive value also in other malignancies. With the rapidly growing amount of high-throughput data from various cancer types, in the future one will be able to study the prognostic value of the signature in other types of neoplastic diseases.

Materials and Methods

Cell lines and nuclear extracts

MCF7, HEK239, HeLa, ZR-75–1, HT-29, U2OS, and Z138 cells were purchased from the American Type Culture Collection (ATCC). UMSCC2 cells were purchased from University of Michigan. NCEB-1 and SP-49 cells were a gift from Dr. Jiri Bartek, the Danish Cancer Society Research Center. H2009 cells were a gift from Novartis. Mouse 3T3 cells were generated as described.[46] All cell lines were maintained in high glucose DMEM with 10% fetal bovine serum (FBS) plus penicillin/streptomycin, except Z138 and U2OS cells that were grown in RPMI1640 and McCoy's medium, respectively, with 10% FBS plus and penicillin/streptomycin,

Tandemly tagged CDK4 was generated by cloning human CDK4 cDNA into *Xho* I and *Not* I sites of pOZ-FH-N expression vector.[47] Nuclear extractions were performed as described,[48] using 2×10^7 cells expressing tagged CDK4 as a source of material. Nuclear extracts were then used for tandem immunoaffinity purification[18] of CDK4-containing complexes. In parallel, we obtained nuclear extracts from cells expressing empty pOZ-FH-N vectors, which were used for control ("mock") purifications.

Mass spectrometry analysis and generation of cyclin D1 and CDK4 interactomes

Cyclin D1 interactome from 5 cancer cell lines, including MCF7 cells, was from our previous report.[18] Mass spectrometry analysis of CDK4 interacting protein partners from MCF7 cells was performed as follows. Purified CDK4-containing complexes from MCF7 cells were subjected to 3 independent mass spectrometry runs (each using a sample containing approximately 300 ng of CDK4). "Mock" purified samples were analyzed in parallel.

Sample preparation for mass spectrometry analysis was as described.[18] A sample that contained at least 300 ng of tagged CDK4 (or the corresponding amount of material prepared from "mock" purified samples) was TCA precipitated, and digested for 5-6 hours at 37°C in a reaction mixture consisting of 50 mM ammonium bicarbonate, pH 8.0, 10 % Acetonitrile (ACN) and 400 ng modified trypsin (Promega). The digestion mixture was quenched with 50 % ACN, 5 % formic acid (FA), and lyophilized to dryness. Dried peptides were then desalted using Empore C18 solid phase extraction disks (3M) as previously described[18]. Samples were resuspended in 5 % ACN, 5 % FA prior to analysis by liquid chromatography and tandem mass spectrometry.

Construction of high-confidence CDK4 and cyclin D1 interactomes

CDK4 interacting proteins were selected as high confidence interacting proteins, if they fulfilled all of the following criteria. (1) The protein was detected by more than 10 independent peptides in CDK4 immunopurifications. (2) The number of spectra seen for this protein in CDK4 immunopurifications was over 20 times higher than that observed in mock purified samples. (3) The protein had a substantially higher probability of being detected in CDK4 immunopurifications than in the "mock" purifications ($p \leq 0.01$ using Chi^2 test).

Using these criteria, we identified 30 high-confidence interactors of CDK4.

The same criteria were previously applied to identify high-confidence cyclin D1 interactors from 5 cancer cell lines,

including MCF7 cells.[18] In our previous report[18] we identified 17 high-confidence interactors for cyclin D1 in MCF7 cells; these interactors are shown in **Fig. 1B.**

Statistical analysis of cyclin D1 interactome

The list of genes for which somatic mutations have been implicated in tumorigenesis was obtained from the COSMIC database (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/) and used to determine the enrichment of cancer-causing genes within the list of high confidence cyclin D1 interacting proteins. In addition, cyclin D1 interacting proteins were annotated for somatic copy-number alterations as determined by Beroukhim et al.[9] Genes within the top 30% of the GISTIC *q*-values were used. Enrichment was determined by Fisher Exact test.

Co-immunoprecipitation and immunodepletion analyses

Cell lysates were prepared in ELB buffer (160 mM NaCl, 50 mM HEPES pH 7.5, 5 mM EDTA pH 8.0 and 0.1% NP-40) supplemented with Roche cocktail proteinase inhibitor. Four μg of mouse anti-cyclin D1 antibody (Ab1 or Ab3, Lab Vision) or mouse anti-CDK4 antibody (Ab1, Lab Vision) were incubated with 5 mg of lysates. Protein G beads were then added, and immunoprecipitated proteins were detected by immunoblotting with the antibodies against: CDK1 (A17.1), from Lab Vision, ZFP106 (A301–527A), FKBP4 (A301–426A), FKBP5 (A301–430A) from Bethyl Laboratories, CDK2 (M2), CDK4 (C-22), CDK5 (DC-17), CDK6 (C-21), CDC37 (H-271) from Santa Cruz Biotechnologies, Flag-M5 (F4042) from Sigma Aldrich, Ras (3965) from Cell Signaling Technology. Anti-β-actin antibody (AC-15) from Sigma or anti-GAPDH antibody from Cell Signaling were used to control for loading.

Immunodepletion was performed similarly to immunoprecipitation except that 10 μg of the indicated antibodies were used. The supernatants were examined by immunoblotting using the indicated antibodies.

Immunoprecipitation-re-immunoprecipitation was performed as follows. MCF7 cells were transfected with p3X Flag-CMV-10-CDK4 mammalian expression vector (E7658, from Sigma-Aldrich). Cell lysates were prepared in ELB buffer. CDK4 was immunoprecipitated using anti-Flag-M2 affinity beads (F2426, Sigma- Aldrich). Immunoprecipitated proteins were eluted by addition of 200 mM of 3X FLAG® Peptide (F7499, Sigma-Aldrich) in ELB and divided into 3 equal parts. The first part was subjected to an anti-FKBP5 immunoprecipitation, the second to an anti-CDC37 immunoprecipitation, and the third to immunoprecipitation with control IgG. Immunoprecipitated complexes were analyzed by immunoblotting using anti-CDK4, FKBP5 and CDC37 antibodies.

Immune complex kinase assays

The immune complex kinase assays were performed as described.[49] Briefly, 10^6 cells were lysed at in 300 μl of ELB buffer supplemented with Roche proteinase inhibitor cocktail, 10 mM β-glycerophosphate, 1 mM NaF, and 0.1 mM sodium orthovanadate (Sigma Aldrich) and sonicated at 4°C. Lysates were incubated with protein G-sepharose beads pre-coated with saturating amounts of anti-CDK4 antibody (Ab1, Lab Vision). The beads were suspended in kinase buffer (50 mM HEPES [pH 7.5], 10 mM MgCl2, 1 mM DTT) containing 0.5 μg of GST-RB fragment as a substrate (sc-4112, Santa Cruz Biotechnologies) plus 2.5 mM EGTA, 10 mMβ-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM NaF, 20 μM ATP, and 10 μCi of γ-^{32}P ATP. After incubation for 30 min at 30°C, the samples were resolved on SDS-PAGE gels, and analyzed autoradiography.

Depletion of FKBP4 and FKBP5

Knock-down of FKBP5 and FKBP4 was performed using 2 independent anti-FKBP5 siRNAs: siFKBP5-A (FKBP5–5, SI02780372) and siFKBP5-B (FKBP5–6, SI02780414), and 2 anti-FKBP4 siRNAs: siFKBP4-A (FKBP4–5, SI02780365), and siFKBP4-B (FKBP4–6, SI02780407). As a control, non-targeting siRNA (Allstars Negative Control siRNA, SI03650318) was used. All siRNAs were from Qiagen.

Ectopic expression of BAIAP2L1

BAIAP2L1 cDNA was obtained by reverse transcription (SuperScript III reverse transcriptase, Invitrogen) from total RNA isolated from U2OS cells, followed by PCR amplification using BAIAP2L1 forward primer: 5′ATATGCGGCCGCAT CCCGGGGCCCGAG3′, and the reverse primer: 5′ATGGTA CCTTCATCGAATGATGGGTGCCGA3′. BAIAP2L1 cDNA was then cloned into p3X FLAG-CMV-10 expression vector. The resulting p3X FLAG-CMV-10 BAIAP2L1 plasmid *was* transfected using Lipofectamine 2000 (Invitrogen). Cells stably expressing BAIAP2L1 were selected with neomycin (500 μg/ml).

Colony formation and soft agar assays

MCF7 or U2OS cells stably overexpressing BAIAP2L1 or transfected with an empty vector were seeded into 6 well plates in triplicate at 100, 200 or 500 cells per well and cultured for 10–12 d. Cells were stained with 0.1% crystal violet. Colonies that contained more than 25 cells were counted.

To generate murine 3T3 cells stably expressing activated v-Ha-Ras, cells were transduced with the retrovirus expressing activated Ras (pBabe-puro-RasV12, Addgene), or for control, with an empty vector. Cells were selected with puromycin (2 μg/ml) for 5 d. To overexpress BAIAP2L1, cells were transfected with p3X FLAG-CMV-10 BAIAP2L1 plasmid as above, and selected in G418 sulfate (500 μg/ml). To perform the soft agar assay, 2 ml of 1.2% noble agar (Becton Dickson) was prepared in DMEM to form the bottom layer of the plate. The top layer of the agar (0.8% noble agar in DMEM) contained cells at the concentration of 5×10^3- 5×10^4 of cells per 2 ml. All visible colonies were counted under 4X-20X magnification after 4 weeks.

***In vivo* tumor growth assays**

All experiments were performed in accordance with the guidelines established by Dana-Farber Cancer Institute's Institutional Care and Use Committee. 4-6 weeks old female nude mice [Nu/Nu (CD-1), from Charles River Laboratories] were injected with

10^6 cells in 0.2 ml PBS and sacrificed 4-8 weeks after injection, when the tumors reached 2 cm in diameter.

Expression of BAIAP2L1 in normal vs. cancer samples

We searched the GENT database,[33] which curates over 40,000 expression profiles measured by Affymetrix U133A or U133plus2 platform for BAIAP2L1 expression. The raw expression levels in cancers vs. in normal samples in 32 tissue types were compared using simple linear regression. Among the 32 tumor types available, only 25 had both cancer and normal samples (the remaining 7 had only tumor samples). These 25 cancer/normal sets were included in the subsequent analysis. The raw expression data were log-transformed and normalized to control for differences in baseline levels between different samples, e.g. cancers vs. normal samples. Then, a random intercept model was used to test for the differences in the expression levels between cancer vs. normal samples in each tissue type.

The difference between cancer vs. normal samples within each tissue type was later evaluated using simple linear regression. Differential expression within the given tissue type was considered statistically significant when $p < 0.002$ (0.05/25), using Bonferroni correction for multiple testing to control for type I error rate.

Construction of cyclin D1 interactome Aggregate Expression Score and survival analysis

Level 3 breast cancer expression data with clinical annotation was obtained from the NIH-TCGA portal (https://tcga-data.nci.nih.gov/tcga/). Patients with ER-positive breast cancers for which with full molecular and survival data was available were used for analyses. We designed the Aggregate Expression Score (AES) to represent overall gene expression level of the interactome as follows:

$$AES = \Sigma_i G_i$$

and

$$G_i \begin{cases} 1 & \text{gene expression} > 1sd \text{ from mean } U \\ -1 & \text{gene expression} < 1sd \text{ from mean } U \\ 0 & \text{Others} \end{cases}$$

where i represents gene i in the interactome, and sd and U indicate the standard deviation and mean gene expression of i, respectively. Cyclin D1 "up" and "down" expression was defined similarly as 1 standard deviation (SD) away from the mean. For every tumor, each of 132 cyclin D1 interactors was assigned a value of +1 (if the expression level of this interactor in this tumor was one SD or more above the mean expression level for this interactor seen in all breast cancer samples), −1 (if the expression level of this interactor in this tumor was at least one SD below the mean expression value for this interactor in all breast cancer samples), or "0" if none of the above 2 criteria were fulfilled. Subsequently, values for all interactors in a given tumor were added up, and AES was obtained. As "high AES," we defined tumors with AES values in the upper 50% across all tumor samples; "low AES" had values are those in the bottom 50%.

Survival curves were estimated using the Kaplan–Meier method for patients partitioned into AES high and AES low groups. The association of AES with survival status was evaluated through a Cox proportional hazard model. All analysis was performed with the R software (http://www.R-project.org).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

This study was supported by Siriraj Research Development Fund and by R01 CA083688 and P01 CA080111 (to P.S.). S.J. is supported by "Chalermphrakiat" Grant and Siriraj Foundation D003421, Faculty of Medicine Siriraj Hospital, Mahidol University and the Thailand Research Fund RSA5580018. Y.E.W is supported through the CCCB and the Dana-Farber Strategic Plan Initiative.

Supplemental Materials

Supplemental data for this article can be accessed on the publisher's website.

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Cyclin D1 Promotes RAD51 Recruitment to the C-Terminus Domain of BRCA2

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Running title: Cyclin D1 promotes RAD51 binding to BRCA2

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 CDK2–cyclin A-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 dislodges cyclin A from the C-terminus of BRCA2, suppresses CDK2–cyclin A-mediated Ser3291 phosphorylation, and facilitates RAD51 binding to the C-terminal domain of BRCA2. These findings indicate that interplay between cyclin D1 and cyclin A cell cycle regulatory proteins regulates DNA integrity through RAD51 interaction with the BRCA2 C-terminal domain.

Keywords: cell cycle/cyclin D1/cyclin A/RAD51–BRCA2/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 (Connor et al, 1997; Wooster et al, 1995). Tumors containing BRCA2 mutants exhibit elevated genomic instability and genetic mutations (Gretarsdottir et al, 1998; Patel et al, 1998; Yu et al, 2000). Several studies established that BRCA2 plays a role in homologous recombination (HR)-mediated DNA repair (Moynahan et al, 2001; Tutt et al, 2001; Xia et al, 2001). A key function of BRCA2 is to mediate loading of RAD51 molecule to single stranded DNA (ssDNA) (Esashi et al, 2007;Jensen et al, 2010; Liu et al, 2010). RAD51 is a recombinase that catalyzes homologous pairing and strand exchange, and thus is a central protein that controls HR (Moynahan & Jasin, 2010). A recent study showed that BRCA2 has a novel function in protecting nascent DNA in the stalled replication fork (gaps of ssDNA) from the endonuclease activity of MRE11 (Schlacher et al, 2011).

BRCA2 functions in these processes by mediating assembly of RAD51 onto ssDNA (resected ends of DNA double-stranded breaks, or replication gaps). 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 gather RAD51 molecules is correlated with its functions. A study by Chen et. al. showed that disruption of BRCA2–RAD51 interactions at the BRC domain significantly reduced RAD51 recruitment to DNA damage foci, and conferred DNA damage hypersensitivity on the cells (Chen et al, 1999). 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 DNA repair defects (Berman et al, 1996; Goggins et al, 1996; Lancaster et al, 1996; Spain et al,

1999). Given the significance, the interactions between RAD51 and BRCA2 are subjected to regulation, especially the C-terminus RAD51 binding domain.

A close relationship between DNA repair and cell division has been recognized. It is established that the mode of repair for damaged DNA is 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 (Jasin & Rothstein, 2013). Cell cycle proteins cyclin-dependent kinases (CDKs) were shown to regulate several steps of cell division, such as activating transcription of S-phase genes, triggering genome replication, and overseeing cytokinesis (Malumbres & Barbacid, 2009) (Deshpande et al, 2005). Deregulation of CDK activities results in DNA damage and genome instability (Cerqueira et al, 2009; Tort et al, 2006). Several reports indicated that cell cycle regulatory proteins also directly control proteins in DNA repair pathways. Several proteins of the HR pathway are substrates for CDKs, including CtIP/SAE2 (Falck et al, 2012; Huertas et al, 2008; Huertas & Jackson, 2009; Wang et al, 2013), NBS1 (Falck et al, 2012), and BRCA2 (Esashi et al, 2005; Yata et al, 2014), underlining a direct involvement of cell cycle proteins in the DNA repair process, especially HR. CDK2–cyclin A 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 (Esashi et al, 2005). The phosphorylation is believed to keep activities of RAD51, and thus HR in check when repair is not required (Esashi et al, 2005). In accordance with this, Ser3291 phosphorylation by CDK2–cyclin A peaks during G2 phase after DNA replication is successfully completed. On the other hand, when DNA is damaged, this phosphorylation event is dramatically downregulated (Esashi et al, 2005), thereby allowing RAD51 recruitment and initiating HR.

Cyclin D1 is a putative cancer-causing protein. Overexpression of cyclin D1 is detected in several human cancers, such as breast cancer (Bartkova et al, 1995b; Buckley et al, 1993;

Gillett et al, 1994), mantle cell lymphoma (Bosch et al, 1994; Komatsu et al, 1993), squamous cell carcinoma (Bartkova et al, 1995a; Jares et al, 1994; Jiang et al, 1992), and colon cancer (Bartkova et al, 1994; Bartkova et al, 1995b), where it is believed to drive cancer cell division and confer chemotherapeutic resistance (Musgrove et al, 2011). Recently, we and others have discovered a novel function of cyclin D1 in HR (Jirawatnotai et al, 2011; Li et al, 2014; Li et al, 2010). Cyclin D1 expression facilitates RAD51 recruitment to DNA damage foci (Jirawatnotai et al, 2012; Jirawatnotai et al, 2011; Li et al, 2010). *In vivo*, cyclin D1 is detected in RAD51-containing DNA damage sites (Jirawatnotai et al, 2011). Suppression of cyclin D1 expression 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 (Jirawatnotai et al, 2011).

Cyclin D1 interacts with RAD51 directly via amino acids 90–155 (Jirawatnotai et al, 2011). 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 (Jirawatnotai et al, 2012). 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

Cyclin D1 interacts with the C-terminal RAD51-binding domain of BRCA2

Previously, using immunoprecipitation coupled with mass-spectrometry, we identified BRCA2 as a cyclin D1-interacting protein (Jirawatnotai et al, 2011). We also determined by *in vitro* binding assay that cyclin D1 directly interacts with BRCA2 (Jirawatnotai et al, 2011). Analyses using fragments of BRCA2 showed that cyclin D1 interaction with BRCA2 is mediated through the

most N-terminus domain of BRCA2 (Jirawatnotai et al, 2011; Lee et al, 2004) (B2-1, Fig 1A), and through two other areas at the C-terminus domain: amino acids 2438–2824 (B2-7, Fig 1A), and 3189–3418 (B2-9, Fig 1A) (Jirawatnotai et al, 2011). 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 co-precipitated with the C-terminal domains B2-7, and B2-9 (Fig 1B). However, unlike the previous *in vitro* GST-binding results (Jirawatnotai et al, 2011), endogenous cyclin D1 marginally co-precipitated with the N-terminal domain of BRCA2 (B2-1) (Fig 1B). The interactions were verified in another cancer cell line, MCF7 (Supplementary Fig S1). These results indicated that endogenous cyclin D1 primarily interacts with the C-terminus of BRCA2. Interestingly, we also found that endogenous cyclin A co-precipitated with all of the BRCA2 fragments that were tested, while cyclin B also co-precipitated with B2-9 (Fig 1B).

To investigate the interaction between cyclin D1 and the BRCA2 domains during the cell cycle, we incubated various BRCA2 fragments in lysates prepared from cells synchronized in G1, S, and G2 phase. We found that endogenous cyclin D1 co-precipitated with the C-terminal fragments of BRCA2 (B2-7 and B2-9) from lysates prepared from cells in G1, S, and G2 phase (Fig 1C). Despite high cyclin D1 expression in G1, and lower cyclin D1 expression in S and G2 phase (Fig 1D), we detected an interaction between cyclin D1 and B2-9 in every phase of the cell cycle. We then performed immunoprecipitation using an anti-BRCA2 antibody in lysate 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 Fig S2). Of note, the interaction between endogenous cyclin D1 and BRCA2 was weaker in cells synchronized in G1, and was upregulated in cells in S and G2 phase (Supplementary Fig S2), implying that the affinity of cyclin D1 towards BRCA2 may be regulated during the cell cycle.

Cyclin A or cyclin B was also tested for their interactions with BRCA2 fragments during each phase of the cell cycle (Fig 1C). We found that endogenous cyclin A interacted with all of the BRCA2 fragments tested, particularly in G2 phase, except for the B2-9 fragment, which cyclin A consistently interacted with in every phase of the cell cycle. Endogenous cyclin B only interacted with C-terminal fragments of BRCA2. Together, these observations support that various cyclins interact with the C-terminal fragments of BRCA2, especially B2-9, *in vitro* and *in vivo*. The differential interactions between cyclins and each of the BRCA2 fragments during each phase of the cell cycle suggested that the interactions are specific and are regulated.

Cyclin D1 suppresses BRCA2 Ser3291 phosphorylation by CDK2

RAD51 was shown to directly interact with the end-most C-terminal fragment of BRCA2 (B2-9) (Davies & Pellegrini, 2007; Esashi et al, 2007; Mizuta et al, 1997). The interaction between RAD51 and B2-9 is abrogated by the phosphorylation of BRCA2 at Ser3291 mediated by CDK2-cyclin A, or when Ser3291 was mutated to glutamic acid (S→E, a phospho-mimicking mutation) (Esashi et al, 2005).

Conversely, the interaction between RAD51 and the C-terminus fragment of BRCA2 was enhanced when Ser3291 phosphorylation was blocked by a CDK2-specific chemical inhibitor, roscovitine (Esashi et al, 2005). These data demonstrated that binding of RAD51 to the C-terminus of BRCA2 is negatively controlled by the kinase activity of a cell cycle protein, CDK2, and prevention of this phosphorylation event enhances RAD51 recruitment to the C-terminus of BRCA2 (Esashi et al, 2005).

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 (Jirawatnotai et al, 2011).

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 (Esashi et al, 2005).

Because the phosphorylation of Ser3291 was shown to be a critical factor that determines RAD51 binding to the C-terminus of BRCA2, and this phosphorylation was associated with cyclin A or cyclin B expression (Esashi et al, 2005), we examined whether cyclin D1 overexpression is associated with Ser3291 hyperphosphorylation.

BRCA2 phosphorylation at Ser3291 was clearly detected by a specific antibody (Esashi et al, 2005) in lysate prepared from asynchronous HeLa cells (FIG 2A, lane 1). As previously reported (Esashi et al, 2005), Ser3291 phosphorylation was highly upregulated when cells were synchronized in G2/M by nocodazole treatment (FIG 2A lane 4, and Fig 2B), and was completely suppressed by roscovitine treatment (FIG 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 (FIG 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 (FIG 2A, B). In agreement with this, cyclin D1 depletion by cyclin D1-specific short-interfering RNAs (siRNAs) enhanced BRCA2 phosphorylation at Ser3291 (FIG 2C).

Given that some CDKs share a common substrate, we investigated if CDK4–cyclin D1 phosphorylates B2-9. We performed *in vitro* CDK4 and CDK2 kinase assays on purified C-terminal domain GST-B2-9. In accordance with a previous report (Esashi et al, 2005), CDK2–cyclin A holoenzyme phosphorylated B2-9 but not B2-5 (B2-5 was used as a negative control) (Supplementary Fig S3). In contrast, although the CDK4–cyclin D1 holoenzyme exhibited strong kinase activity toward a C-terminal fragment of pRB (used as a positive control),

phosphorylation of GST-B2-9 by CDK4–cyclin D1 was undetectable (Supplementary Fig S3). Therefore, we concluded that the C-terminal fragment of BRCA2 (B2-9), while a suitable substrate for CDK2–cyclin A, is not a substrate for cyclin D1 and its associated kinase partner CDK4.

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 (Fig 2D, lane 2). Under this condition, the Ser3291 phosphorylated B2-9 fragment co-precipitated with cyclin A and a small amount of RAD51 (Fig 2D, lane 2). When incubated in lysate prepared from cells treated with roscovitine however, phosphorylation at Ser3291 on B2-9 was significantly suppressed (Fig 2C, lane 4). Inhibition of Ser3291 phosphorylation by roscovitine was associated with increasing amounts of RAD51 co-precipitated with B2-9 (Fig 2C; lane 4 compared with lane 2).

When incubated in lysates prepared from cells ectopically expressing cyclin D1, Ser3291 phosphorylation on B2-9 became undetectable (Fig 2C, lane 3). Under this condition, we observed that the B2-9 interaction with RAD51 was greatly enhanced (Fig 2C, lane 3), while the interaction with cyclin A was significantly reduced (Fig 2D, lane 3). We also observed that cyclin D1 clearly co-precipitated with the fragment (Fig 2D, 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 that were composed of purified HA-tagged-cyclin D1 and GST-B2-9.

Compared with cyclin D1, cyclin A was a weaker competitor for B2-9 binding (Fig 3A, 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 (Fig 3A, B).

In a converse 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 better 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 Fig 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 (Esashi et al, 2005). DNA damage was demonstrated to suppress phosphorylation at this moiety (Esashi et al, 2005) (Fig 4A). Upon subjection to ionizing radiation (IR), we found that binding of cyclin A to the C-terminus BRCA2 fragment was significantly reduced (Fig 4A). Interestingly, IR treatment significantly enhanced binding of cyclin D1 to the C-terminal B2-9 fragment of BRCA2 (Fig 4A).

We then analyzed Ser3291 phosphorylation on endogenous BRCA2 by immunoblotting. Again, we found that nocodazole treatment enhanced BRCA2 Ser3291 phosphorylation to a level that was much higher than that of untreated cells (Fig 4B, lane 2 compared with lane 1). Roscovitine suppressed Ser3291 phosphorylation, confirming that it was CDK2-specific phosphorylation (Fig 4B, lanes 3, 4). Ectopic expression of cyclin D1 or DNA damage suppressed Ser3291 phosphorylation in both nocodazole-treated and -untreated cells (Fig 4B, lanes 5, 6, and lanes 7, 8, respectively). Cyclin D1 overexpression and IR treatment cooperated to further suppress

BRCA2 Ser3291 phosphorylation completely, both in untreated and nocodazole-treated cells (Fig 4B, lane 9, 10).

It has been demonstrated that DNA damage can trigger the degradation of cyclin D1 (Agami & Bernards, 2000; Lin et al, 2006). We examined the expression levels of cyclin D1 and cyclin A following DNA damage caused by a moderate dose of IR (5 Gy). As previously demonstrated (Agami & Bernards, 2000; Lin et al, 2006), we confirmed that cyclin D1 expression was downregulated after DNA damage. Cyclin D1 level was downregulated to 50% at 6 h after IR treatment (Fig 3C, D). Cyclin D1 expression remained low but was not entirely ablated by the treatment. On the other hand, cyclin A appeared to be more sensitive to IR and was reduced to 50% by the same dose of IR at 2 h after treatment (Fig 3C, D). At 6 h after treatment, cyclin A expression had virtually disappeared (Fig 3C, D). Therefore, under DNA damaging conditions, cyclin D1 expression persisted, while cyclin A expression was severely repressed. This context is favorable for cyclin D1 binding to the C-terminus of BRCA2.

To investigate the possibility that cyclin D1 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 Fig S5, lane 6).

Increasing the amount of cyclin D1 in the reaction gradually increased cyclin D1 binding to B2-9 (Supplementary Fig S5, 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 Fig S5, lanes 7–10).

These results indicated that cyclin D1 alone does not facilitate RAD51 recruitment to the C-terminus of BRCA2. However, the role of cyclin D1 in RAD51 recruitment is to prevent the inhibitory Ser3329 phosphorylation event caused by CDK2–cyclin A.

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 Δ20–295 that lacks amino acids 1–19 at the N-terminus of cyclin D1, and cyclin D1 Δ91–295 that lacks amino acids 1–90 (Fig 5A). We tested the mutants in an *in vitro* binding assay. We found that purified full-length cyclin D1 and cyclin D1 Δ20–295 were able to interact with the B2-9 fragment of BRCA2 (Fig 5B, lane 2, 3), therefore amino acids 1–20 of cyclin D1 were not required for binding to the C-terminus of BRCA2. The mutant cyclin D1 Δ91–295 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 (Fig 5B lane 4). In accordance with this, while purified full-length cyclin D1 prevented B2-9 phosphorylation caused by CDK2–cyclin A holoenzyme in an *in vitro* kinase assay, mutant cyclin D1 Δ91–295 did not prevent phosphorylation as efficiently as the full-length protein (Fig 5B, C).

We noticed that the BRCA2-binding domain (amino acids 20–90), and the RAD51 binding domain (amino acids 90–155) we previously reported (Jirawatnotai et al, 2011) coincidentally overlap with the well-described CDK-binding domain (cyclin box and extended area in N-terminus of cyclin D1, amino acids 40–170) (Zwicker et al, 1999). Therefore, we hypothesized that cyclin D1 molecules that are in complex with CDK4 are not able to function to prevent BRCA2 phosphorylation by CDK2–cyclin A. We investigated this by *in vitro* kinase assays. We observed that, unlike unbound cyclin D1 (Fig 5C), cyclin D1 in complex with CDK4 (CDK4–cyclin D1) failed to prevent CDK2-dependent B2-9 phosphorylation (Supplementary Fig S6).

Interplay between cyclin D1 and cyclin A regulates BRCA2 Ser3291 phosphorylation *in vivo*

To study the role of the interplay between cyclin D1 and cyclin A 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 (Jirawatnotai et al, 2011). 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 (Fig 6A). HeLa cells do not contain functional pRB, therefore, expression of cyclin D1 is not required for proliferation of these cells (Bates et al, 1994; Lukas et al, 1995). Accordingly, depletion of cyclin D1 did not alter the cell cycle profiles of these cells (Supplementary Fig S7).

In control cells expressing non-target shRNA, we found that BRCA2 Ser3291 phosphorylation was downregulated during G1 to S-phase when cyclin D1 expression was relatively high. Ser3291 phosphorylation peaked when cells entered G2 phase at 4 h after release. The heightened level of Ser3291 phosphorylation was correlated with elevated expression of cyclin A and cyclin B.

Interestingly, cyclin D1 depletion abolished the suppression of Ser3291 phosphorylation during G1 and S phase. As a result, we detected Ser3291 hyperphosphorylation during every phase of the cell cycle. Therefore, Ser3291 phosphorylation is regulated by the relative expression of cyclin D1 and A.

Discussion

Several reports indicated that the pathways that regulate the cell cycle and DNA repair are collaborative. A number of cell cycle regulatory proteins were uncovered as crucial factors for DNA repair (Lim & Kaldis, 2013; Wohlbold & Fisher, 2009; Yata & Esashi, 2009). Previously, we identified cyclin D1 as an important protein required for RAD51 recruitment to BRCA2-positive DNA repair foci and efficient HR repair (Jirawatnotai et al, 2011). Here, we elucidated a possible mechanism employed by cyclin D1 to promote the recruitment of RAD51 to BRCA2. We found

that, via amino acids 20–90, cyclin D1 interacts directly with the C-terminus of BRCA2 at amino acids 3189–3418, and suppresses the negative regulation of RAD51 binding on BRCA phosphorylation at Ser3291 caused by CDK2–cyclin A. According to this view, cyclin D1 alone did not enhance RAD51 binding to B2-9. Instead, cyclin D1 indirectly facilitated RAD51 recruitment and HR-mediated DNA repair by fencing off the inhibitory phosphorylation caused by CDK2–cyclin A.

When RAD51 recruitment to the C-terminal domain of BRCA2 is not required, Ser3291 is highly upregulated by CDK2–cyclin A or B activity (Fig 6A, B). However, under circumstances where RAD51 recruitment to the C-terminal domain of BRCA2 is required, Ser3291 phosphorylation has to be downregulated. Under one such condition, such as after DNA damage, we observed that cyclin D1 downregulated this phosphorylation event. HR-mediated DNA repair is normally the main repair mechanism for mammalian cells in S or G2 phase of the cell cycle. During these phases, expression of cyclin A, as well as activity of CDK2, is upregulated (Elledge et al, 1992; Pagano et al, 1992; Pines & Hunter, 1990; Rosenblatt et al, 1992). As a result, Ser3291 becomes hyperphosphorylated and RAD51 recruitment to C-terminal domain of BRCA2 is inhibited (Esashi et al, 2005). Although cyclin D1 may be an intrinsically stronger competitor for BRCA2 binding, a relatively higher level of cyclin A could out-compete cyclin D1 (Fig 6B, left). However, in conditions where RAD51 recruitment at the C-terminus of BRCA2 is required (DNA damage), cyclin A is rapidly degraded while cyclin D1 remains at a low level. This allows cyclin D1 to dislodge cyclin A from BRCA2, thus Ser3291 becomes hypophosphorylated and RAD51 binding to the C-terminus of BRCA2 is increased (Fig 6B, right).

Interestingly, DNA damage seemed to enhance the interaction between cyclin D1 and BRCA2 (Fig 4A). Despite this, cyclin D1 protein was significantly downregulated by IR, and an increasing amount of the protein was co-precipitated with the C-terminus of BRCA2. This finding implied that there might be post-translational modification(s) triggered by IR that promotes the

interaction of cyclin D1 with BRCA2. Several detailed studies have described DNA damage-independent and -dependent cyclin D1 modifications (Hitomi et al, 2008; Lin et al, 2006; Pontano et al, 2008; Santra et al, 2009; Sewing & Muller, 1994). These modifications typically trigger cyclin D1 degradation and cell cycle arrest. It is tempting to speculate that these modifications enhance the BRCA2-binding efficiency of cyclin D1.

It is possible that there are two different pools of cyclin D1. One has a role in driving the cell cycle, while another plays a role in HR. Some post-translational modifications may prioritize which role to undertake or when to perform it. In addition, our results showed that the BRCA2- and RAD51-binding domains of cyclin D1 almost completely overlap with the CDK4-binding domain (cyclin box) (Zwicker et al, 1999). Thus, CDK4-bound cyclin D1 might not be able to bind to BRCA2 or RAD51. As a result, it does not inhibit CDK2 phosphorylation at the C-terminus of BRCA2 (Supplementary Fig S6). Therefore, the choice of partner would also determine the role of cyclin D1.

RAD51 recruitment to the C-terminal domain of BRCA2 is required for BRCA2 function. Previously, we demonstrated that cyclin D1 facilitates HR-mediated DNA repair, and cyclin D1 depletion attenuates HR-mediated DNA repair and cancer cell survival after camptothecin, etoposide, or IR treatment (Jirawatnotai et al, 2011). Recently, two groups elegantly revealed that BRCA2 and RAD51 function in blocking stalled replication fork degradation caused by MRE11 (Hashimoto et al, 2010; Schlacher et al, 2011). In one study, the C-terminal RAD51-binding domain was shown to be essential for this novel function (Schlacher et al, 2011). Whether or not cyclin D1 participates in this new function remains to be determined.

We previously reported that cyclin D1 facilitates RAD51 recruitment to the BRCA2-containing DNA repair foci (Jirawatnotai et al, 2011). In this study, we only focused on a possible mechanism by which cyclin D1 promotes RAD51 recruitment to C-terminus of BRCA2.

However, the involvement of cyclin D1 regarding RAD51 recruitment to other areas such as the BRC repeat on BRCA2 is still unclear.

Materials and Methods

Cell lines and synchronization

HeLa and MCF7 cells were purchased from American Type Culture Collection (ATCC). Cells were maintained in high glucose DMEM (Invitrogen) supplemented with 10% fetal bovine serum and penicillin/streptomycin. When required, roscovitine was used at a final concentration of 10 μM. HeLa cells were synchronized in G1 phase by treatment with 20 μM lovastatin for 33 h according to a previous protocol (Javanmoghadam-Kamrani & Keyomarsi, 2008). Cells were synchronized in S phase by double thymidine block; cells at 60% confluency were treated with 2 mM thymidine for 20 h, released for 8 h, then treated again with 2 mM thymidine for 20 h and released for 2.5 h before harvesting. Cell synchronization in G2 phase was achieved by 24 h treatment with 50 μg/L nocodazole. For cell cycle re-entry, cells were synchronized by double thymidine block. Cells were harvested every 1 h. Cell cycle analysis was performed using propidium iodide.

Production of recombinant proteins and binding assays

Production of recombinant cyclin D1, cyclin A, and deleted mutants was performed according to a protocol described previously (Jirawatnotai et al, 2011). Briefly, to construct GST-fusion proteins, human cyclin D1, cyclin A, and deletion mutants were subcloned into pGEX-5x-3 (GE Healthcare). Constructs encoding GST-fragments of BRCA2 (Lee et al, 2004) were kindly provided by Dr. A. Venkitaraman, University of Cambridge. Protein expression was carried out for 20 h at 20 °C in *E. coli* BL21 Rosetta strain (Novagen) induced by 0.4 mM IPTG at a culture density of OD600 = 0.6. Bacteria from 1 L culture were harvested by centrifugation (5000 × *g*, 20 min, 4 °C), resuspended in 50 ml lysis buffer (50 mM Tris-HCl pH 7.5, 0.1 % Triton X-100, 1

mM DTT, 2 mM EDTA, 0.1 mg/ml lysozyme, 0.5 mM PMSF, protease inhibitor cocktail) and sonicated. After centrifugation (20,000 × *g*, 20 min, 4 °C) the supernatant was incubated (2 h at 4 °C) with 0.5 ml GSH Sepharose (GE Healthcare). The resin was next washed with 20 ml of PBS supplemented with 250 mM KCl, 1 mM DTT, 0.1 % Tween-20, 0.5 mM PMSF, then with 10 ml of PBS with 1 mM DTT. Proteins were eluted with 3 × 0.5 ml of elution buffer (50 mM Tris-HCl pH 8.1, 150 mM KCl, 1 mM DTT, 10 mM reduced glutathione). When required, the GST-tag was removed by incubating the beads (20 h, 20 °C) with 30 U Factor Xa (Novagen). The proteins were concentrated with buffer exchange (20 mM Hepes pH 7.5, 0.1 mM KCl) using SpinX UF Concentrators (Corning), supplemented with 10 % glycerol, aliquoted and frozen in liquid nitrogen. *In vitro* binding was performed as described (Esashi et al, 2005) 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). Next, 100 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. To identify the BRCA2-interacting region of cyclin D1, 10 pmoles of each cyclin D1 deletion mutant was mixed with GST-B2-9. After binding, beads were washed four times with 0.5 ml of ice-cold binding buffer, resuspended in 20 µl of SDS-PAGE sample buffer and boiled. Proteins were separated using 12 % SDS-PAGE gels and analyzed by immunoblotting using cyclin D1- and GST-specific antibodies.

GST pull-down of endogenous cyclins and co-immunoprecipitation

Lysates were prepared from HeLa cells at 80% confluency. The lysates 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 µg of each GST fusion protein was incubated overnight at 4 °C in 1 mg of lysate. GST-BRCA2 fragments were pulled down using 20 µl of GSH Sepharose and washed five times 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 where phosphorylation of GST-B2-9 was to be examined, pull-down experiments were performed in kinase buffer (described below) without the addition of γ-^{32}P ATP. Co-immunoprecipitation of endogenous BRCA2 and cyclin D1 was performed using a monoclonal antibody specific to BRCA2 (ab1, EMD Millipore), and cyclin D1 immunoblotting was performed using rabbit anti-cyclin D1 antibody (H295) (Santa Cruz Biotechnologies).

Cyclin D1/cyclin A competition assay

Competition assays were performed according to a previously described protocol (Thorslund et al, 2007). 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. GST-B2-9 and the interacting proteins were pulled down using 10 µl 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 in kinase buffer (50 mM HEPES [pH 7.5], 10 mM MgCl2, 1 mM DTT). Each reaction contained 0.5 µl of purified active CDK4–cyclin D1 kinase (ProQinase, Germany), 0.5 µg of GST-RB fragment (Santa Cruz Biotechnologies) or GST-B2-9 as substrates, and 2.5 mM EGTA, 10 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM NaF, 20 µM ATP, and 10 µCi of γ-^{32}P ATP. CDK2–cyclin A kinase assays were performed similarly, except that CDK4–cyclin D1 was replaced with active CDK2–cyclin A kinase (Millipore). B2-5 was added as a substrate for the negative control. In the competition assay, increasing concentrations of recombinant proteins (cyclin D1, cyclin D1Δ91–295, or CDK4–cyclin D1) at 10 nM, 20 nM, or 40 nM were added to the reaction. After incubation for 30 min at 30 °C, the samples were terminated with SDS-PAGE running buffer, and the kinase products were resolved on SDS-PAGE gels and analyzed by autoradiography.

siRNA, shRNA and antibodies

Cyclin D1-specific siRNA sequence A (siD1-A, 5'-CCAAUAGGUGUAGGAAAUAGCGCTG-3') was from Integrated DNA Technologies. Cyclin D1-specific siRNA sequence B (siD1-B, 5'-AACACCAGCTCCTGTGCTGCG-3') and C (siD1-C, 5'-GCCCTCGGTGTCCTACTTCAA-3'), control siRNA (sicont, AllStars Negative control) were from Qiagen. Cyclin D1-specific shRNA (5'-GCCAGGATGATAAGTTCCTTT-3'), and non-target control shRNA (5'-CAACAAGATGAAGAGCACCAA-3') were from Sigma. The following antibodies were used: anti-cyclin D1 antibody (H295, Santa Cruz Biotechnologies), antibody raised against the C-terminus of cyclin D1 (Ab3, Lab Vision), anti-BRCA2 antibody (ab1 OP-95, Merck), anti-RAD51 antibody (H-92, Santa Cruz Biotechnologies), anti-cyclin A (C-19, Santa Cruz Biotechnologies), anti-cyclin E antibody (M-20, Santa Cruz Biotechnologies), anti-GST antibody (Z-5, Santa Cruz Biotechnologies), anti-HA antibody (12CA5, Covance), anti-CDK4 antibody (C-22, Santa Cruz Biotechnologies), anti-β actin (AKR-002, Sigma), anti-GAPDH antibody (AKR-001, Sigma). Anti-phospho-Ser3291 BRCA2 antibody was described previously(Esashi et al, 2005).

Acknowledgements

We thank Dr. Piotr Sicinski from the Dana-Farber Cancer Institute for his valuable comments on this manuscript. This study was supported by the Thailand Research Fund RSA5580018, Siriraj Research Fund, Faculty of Medicine Siriraj Hospital, Mahidol University, and the Advanced Research on Pharmacology Fund, Siriraj Foundation. SJ was supported by the Chalermphrakiat Grant, Faculty of Medicine Siriraj Hospital, Mahidol University.

Author contributions

SJ conceived the research, and interpreted the data. *In vitro* binding assays were performed by SJ, and WM. Recombinant proteins were constructed and produced by WM. *In vitro* competition

assays were performed by SJ. Cell cycle synchronizations, RNAi, and immunoblotting were performed by CC, and GS under the supervision of SJ. Kinase assays were performed by SJ. SJ designed the experiments and directed the study with the help of FE. CC, GS, and SJ prepared the figures and wrote the manuscript.

Conflict of interest

The authors have no conflicts of interest to declare.

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Figure legends

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, B2-5, B2-7, B2-9 (Lee et al, 2004). 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, B2-7, 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)** Interactions between GST-BRCA2 fragments and cyclins in G1, S, and G2 phase of the cell cycle. GST-BRCA2 fragments were incubated with lysates prepared from HeLa cells synchronized in G1, S, and G2 phase. Co-precipitated cyclins were analyzed using specific antibodies. **D)** Immunoblotting of cyclin A, B, and D1 expression in lysates synchronized in G1, S, and G2 phase used in **(C)**. AS; asynchronous. GAPDH was used as a loading control.

Figure 2. Cyclin D1 suppresses S3291 phosphorylation on BRCA2 and facilitates RAD51 binding to the C-terminus of BRCA2. (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 roscovitine (Ros). Lanes 1–3 contained lysates prepared from asynchronous cells, while lysates in lanes 4–6 were prepared from nocodazole-treated cells. Expression of cyclin A, B, D1, CDK4, BRCA2, and RAD51 were analyzed as indicated. **B)** Cell cycle distribution of HeLa cells with indicated treatments. **C)** Immunoblot (IB) analyses of phospho-Ser3291 BRCA2 (S3291 Ph) in HeLa cells treated with cyclin D1-specific small interfering RNA (siD1), HeLa cell ectopically expressing cyclin S1 (D1), ionizing radiation (IR), and roscovitine (Ros). **D)** 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 (lane 4). Co-precipitated proteins were analyzed using the specific antibodies indicated

Figure 3. 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 a GST-specific antibody. **B)** Percentages of HA–cyclin D1 bound to B2-9 in the presence of purified cyclin D1 or cyclin A.

Figure 4. Cyclin D1 cooperates with DNA damage to inhibit BRCA2 phosphorylation at S3291. A) Co-precipitation of cyclin A and cyclin D1 after 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 phosphor-Ser3291 under various treatments were analyzed by immunoblotting. The treatments included nocodazole, ionizing radiation (IR), ectopic cyclin D1 expression (D1), and roscovitine (Ros). Expression of BRCA2, cyclin D1 and CDK4 were also analyzed. Actin was used as a loading control. **C)** Immunoblots (IB) indicate expression levels of cyclin A and cyclin D1 at various time-points after IR treatment. **(D)** Percentage of cyclin A and cyclin D1 protein levels after IR treatment.

Figure 5. 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 (Δ20–295 and Δ91–295). Light grey color highlights indicate known functional domains of cyclin S1, such as pRB binding domain, cyclin box, and pest sequence (Zwicker et al, 1999).**B)** *In vitro* binding assays using GST–BRCA2 B2-9 and purified full-length cyclin D1 (aa1–295) or the indicated cyclin D1 deletion 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 negative control for pull-downs. **C)** B2-9 phosphorylation by CDK2 was efficiently inhibited by full-length cyclin D1, but not by Δ91–295 mutant. *In vitro* CDK2 kinase assays were performed with increasing amounts (0nM, 10nM, 20nM, 40nM) of either purified cyclin D1 or Δ91–295. Kinase activities were analyzed by autoradiography of ^{32}P transferred to B2-9 by CDK2–cyclin A holoenzyme. Immunoblotting was performed to verify levels of GST–B2-9 and purified cyclin D1 and Δ91–295, using a GST-specific antibody and a cyclin D1-specific antibody. **D)** Relative densities of the signals from **(C)**. **E)** Diagrams depict cyclin D1 domains involved in cell cycle function (top), and those domains participating in BRCA2 and RAD51 binding (bottom).

Figure 6. Cyclin D1 regulates BRCA2 S3291 phosphorylation *in vivo*. A) Immunoblot (IB) analyses of phospho-Ser3291 BRCA2 (S3291 Ph), BRCA2, and cyclins during the cell cycle. Lysates were prepared from HeLa cells expressing cyclin D1-specific short hairpin RNA (shcyclin D1), or non-target short hairpin RNA (shcont). GAPDH was used as a loading control. **B)** Phosphorylation of Ser3291 by CDK2–cyclin A when RAD51 recruitment to C-terminus of BRCA2 was not required (Esashi et al, 2005) (left). Cyclin D1 promotes RAD51 recruitment to

the C-terminus of BRCA2 by dislodging cyclin A from BRCA2, thus inhibiting Ser3291 phosphorylation (right).

The Joint 7th AOHUPO Congress and 9th International Symposium of the Protein Society of Thailand

9th PST Symposium

Co-Hosted by Chulabhorn Research Institute

Date: 6-8th August 2014 **Venue: Miracle Grand Convention Hotel, Bangkok, Thailand**

Frontiers in Protein and Proteomic Research

About the Event

The Joint 7th AOHUPO Congress and 9th International Symposium of the Protein Society of Thailand (7th AOHUPO/9th PST), hosted by the Protein Society of Thailand and Chulabhorn Research Institute, will be held at the Miracle Grand Convention Hotel, Bangkok, Thailand during 6-8 August, 2014. The meeting aims to draw attention from protein researchers globally, with the anticipated attendance of 500-600 participants.

The scientific program is being worked out. Briefly, there will be a number of interesting Plenary Lectures by Nobel Laureate as well as some 30 renowned Invited Speakers from overseas and 10-15 leading Thai researchers. In addition to the stimulating scientific program, participants can enjoy visiting exhibition booths from companies whose products are directly involved with protein research.

Topics

- Quantitative Proteomics and Novel Techniques
- Biomarker Discovery
- Drug Discovery and Chemical Proteomics
- Enzymatic Catalysis
- Post-translational Modifications
- Bioinformatics, Systems Biology and Omics Technology
- Food, Agriculture & Energy
- Communicable Disease and Microbial Proteomics
- Non-communicable Disease
- Biotechnology and Protein Applications
- Young Scientist Protein Workshop

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Welcome Message

Dear Colleagues,

On behalf of the Organizing Committee, I have the honor and privilege to invite you to attend the Joint 7th AOHUPO Congress and 9th International Symposium of the Protein Society of Thailand (7th AOHUPO/9th PST), which will be held at the Miracle Grand Convention Hotel, Bangkok, Thailand during 6-8 August, 2014.

The 7th AOHUPO/9th PST will focus on vast areas of protein and life science research. The program will include Plenary Lectures by Nobel Laureate and other top protein scientists, some 30 Invited Speakers from overseas, and 10-15 leading Thai researchers. There will be Poster Sessions for registered participants, and exhibition of companies whose products are relevant to protein research. We anticipate that there will be 500-600 participants in this meeting, and feel sure that you will enjoy the Scientific Program and Social Activities.

I therefore would like to invite you to join the 7th AOHUPO/9th PST and look forward to welcoming you all in Bangkok in 2014.

Sincerely yours,
Professor M.R. Jisnuson Svasti
President, The Protein Society of Thailand

List of Accepted Speakers (more to be invited)

Plenary Lecturers

Ada Yonath
Nobel Laureate in Chemistry 2009, Weizmann Institute of Science, Israel

William S. Hancock
Northeastern University
USA

Naoyuki Taniguchi
RIKEN, Osaka University
Japan

John Yates III
The Scripps Research Institute
USA

Invited Speakers

Yu-Ju Chen	Taiwan
Maxey C.M. Chung	Singapore
Fuchu He	China
Hisashi Hirano	Japan
Bill Jordan	New Zealand
Ajay Kohli	Philippines
Ho Jeong Kwon	Korea
Pao-Chi Liao	Taiwan
Kazuyuki Nakamura	Japan
Young-Ki Paik	Korea
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The cyclin D1-CDK4 oncogenic interactome enables identification of potential novel oncogenes and clinical prognosis

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Genes encoding cyclin D1, and its catalytic partner CDK4, represent the second and the fourth most frequently mutated loci in the human cancer genome. Using an affinity purification coupled with LC/LC/MS, we have constructed a cyclin D1 and CDK4 interaction network from the human breast cancer cell line MCF7. Within this network, cyclin D1 and CDK4 largely interact with different sets of partners. While the majority of cyclin D1 interacts with CDK4, the bulk of CDK4 interacts with proteins functioning in protein folding and complex assembly, namely heat shock proteins and chaperonins. Among CDK4 interactors, we identified FKBP5 as a novel CDK4 protein partner that is required to maintain CDK4 levels in cancer cells. When an extended cyclin D1 interactome was overlaid with a database of genes amplified/deleted in human cancers, a potential oncogene, BAIAP2L1, was identified. Lastly, we derived an Aggregate Expression Score (AES) which integrates the expression levels of all cyclin D1 interactors in human breast cancers. We observed that AES of cyclin D1 interactors has a prognostic value among patients with ER-positive breast cancers. These studies illustrate the utility of analyzing the interactomes of proteins involved in cancer to uncover potential oncogenes, or to allow better cancer prognosis.

Supported by Siriraj Research Development Fund and by R01 CA083688 and P01 CA080111. S.J. is supported by "Chalermphrakiat" Grant, Faculty of Medicine Siriraj Hospital, Mahidol University and the Thailand Research Fund RSA5580018. Y.E.W is supported through the CCCB and the Dana-Farber Strategic Plan Initiative. All the authors declared no conflict of interest.

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Proceedings of the 37th Congress on Pharmacology of Thailand

Genomic Medicine and Novel Cancer Therapy:
Challenges and Opportunities"

May 28-30, 2015

Sunee Grand Hotel & Convention Center, Ubon Ratchathani

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Faculty of Pharmaceutical Sciences, Ubon Ratchathani University

Cancer Targeted Therapy

Siwanon Jirawatnotai, Ph.D.

Cancer targeted therapy is a novel paradigm, and a promising hope for cancer treatment. Accumulated research on the steps that contribute to the tumorigenesis, e.g. immortalization, cellular transformation, angiogenesis, and invasion and metastasis, has resulted in specific sets of gene that apparently can be proposed as suitable targets for cancer treatment. Currently, more than 100 cancer genes are labeled as "actionable" for treatment. With advance in cancer research, a lot more genes will be identified and used as a drug target, in a near future. However, drug resistance and short-term effectiveness are seriously undermining this new paradigm. The shortcomings are supposedly a result of the lack of the "larger picture" of how cancer is operating. We will discuss the usefulness, and the problem of the targeted therapy, hoping to better understand the so-called "magic bullet".