Regulation of Rad17 Protein Turnover Unveils an Impact of Rad17-APC Cascade in Breast Carcinogenesis and Treatment*2

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Background: Abrogated Rad17-APC axis is involved in genome instability and tumorigenesis.

Results: Proteolytic regulation of Rad17 is involved in breast tumorigenesis and could be targeted for chemosensitization.

Conclusion: Regulation of Rad17 turnover is important in the maintenance of genome stability and carcinogenesis.

Significance: Demonstration of impact of Rad17 turnover regulation in genome stability, tumorigenesis, and chemosensitization.

Aberrant regulation of DNA damage checkpoint function leads to genome instability that in turn can predispose cellular tissues to become cancerous. Previous works from us and others demonstrated the role of Rad17 in either activation or termination of DNA damage checkpoint function. In the current study, we revealed the unexpected accumulation of Rad17 in various types of breast cancer cell lines as well as human breast cancer tissues. We observed that Rad17 protein turnover rate in breast epithelial cells is much faster than in breast cancer cells, where the turnover of Rad17 is regulated by the Cdh1/APC pathway. We further observed that Rad17-mediated checkpoint function is modulated by proteolysis. Stabilization of Rad17 disrupts cellular response to chemotherapeutic drug-induced DNA damage and enhances cellular transformation. In addition, manipulation of Rad17 by RNA interference or stabilization of Rad17 significantly sensitize breast cancer cell to various chemotherapeutic drugs. Our present results indicate the manipulation of Rad17 proteolysis could be a valuable approach to sensitize breast cancer cell to the chemotherapeutic treatment despite of the critical role in governing DNA damage response and cellular recovery from genotoxic stress.

Recent studies using clinical specimens from different stages of human tumors have indicated that loss of genomic integrity due to defective ATR/ATM-DNA damage response network contributes to tumorigenesis (1). Targeting the circuitry of DNA damage responses and DNA repair has been a focus in the development of anti-cancer drugs as well as novel cancer therapeutic strategies (2). DNA damage response system surveils the DNA damaged lesion, amplifies DNA damage signaling, recruits DNA repair components to damaged lesion, activates DNA damage checkpoint, and arrest cell cycle progression, which guards genomic integrity. Aberration in DNA damage response could lead to disrupted genome stability, which in turn, predispose normal cell to become cancerous. Previous work from us and other groups revealed that Rad17 is a critical node in the DNA damage signaling pathway and is involved in either activation or deactivation of DNA damage checkpoint signaling (3–5). Dysregulation of Rad17 is found associated with cancer status in various types of tumors (6–9). Thus, we explored whether dysregulation or aberration of Rad17 is a causal factor that contributes to tumorigenesis. Using cultured breast cancer cells, we have examined the impact of Rad17 in breast carcinogenesis and further tested the role of Rad17 in chemosensitization of breast cancer cell.

Rad17 was initially identified and characterized as a DNA damage checkpoint protein (10, 11). Genetic targeting deletion of Rad17 in mouse displayed embryonic lethality with numerous developmental defects (12). Knock-out of Rad17 using somatic gene deletion approach in HCC116 and DT40 cells inhibits mitotic as well as S phase damage checkpoint functions and further results in chromosomal aberration and endoreduplication (13, 14). Biochemical study of Rad17 involvement in the activation of DNA damage checkpoints in human cell has determined functional relationships between Rad17 and ATR. ATR in response to DNA damage phosphorylates Rad17 at two SQ motifs (Ser-635 and Ser-645) near the C terminus (4, 15, 16). Rad17 phosphorylation promotes loading of the checkpoint complex 9-1-1 at DNA damage lesions, which potentially creates a chromatin location site for the 9-1-1 sliding clamp to

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meet other elements of the checkpoint machinery such as ATR, ATRIP, clasin, and Chk1 (4, 17, 18). A recent study showed that Rad17 could present clasin, which bridges Chk1 to ATR, resulting in the phosphorylation and activation of Chk1 (17).

We explored the mechanism involved in the dysregulation of DNA damage response that contributes to tumorigenesis. Results of tissue array based on a variety of tumor specimen unraveled the correlation between defects of DNA damage response machinery, including DNA damage sensors, signaling kinases, and diverse effectors involved in DNA repair, cell cycle checkpoints, chromatin remodeling, and cell death pathways with tumor conditions (1, 19). These observations suggest that DNA damage response/DNA repair could be a barrier for gene mutation accumulation and carcinogenesis (1, 20). We asked whether aberration of Rad17, a pivotal checkpoint regulator, is involved in cancer development. It has been reported that accumulation of Rad17 is detected in a broad range of cancer tissues and cell lines, including lung, breast, and colon cancers as well as G361, A549, MOLT-4, HeLa S3, HLL0, MCF-7, MRM-1, HTB58, CRL185, and HTB119 (6–9). Yet, how does deregulation of Rad17 in involved in cancer formation still remains unclear. DNA damage and repair have been studied as mechanisms for design of anti-cancer drugs or sensitization of cancer cells in combination with chemotherapeutic drugs. Recent studies of VE-821 (ATR specific inhibitor), KU-55933 (ATM inhibitor), UCN-01 (Chk1 inhibitor), and olaparib (poly-ADP ribose polymerase inhibitor) shed a light on cancer treatment through the view of DNA damage response (21–24). In breast cancer, severe unexpected accumulation of several checkpoint proteins such as Rad17, Chk1, and poly-ADP ribose polymerase 1 was documented. Given the notion that Rad17 is tightly regulated by the ubiquitin-proteasome pathway in fibroblast or cervical cancer cell, we studied whether Rad17 is regulated by the protein turnover and whether manipulation of Rad17 protein stability could sensitize breast cancer cell to genotoxic drugs (3). Overall, our present study of Rad17 and its regulation by ubiquitin-proteasome system in breast carcinogenesis and chemosensitization unveiled a new aspect of Rad17 regulation by ubiquitin-proteasome system in breast carcinogenesis. It has been reported that whether aberration of Rad17, a pivotal checkpoint regulator, is involved in breast cancer, severe unexpected accumulation of several checkpoint genes (240 and 243) by site-directed mutagenesis. Was generated by replacement of arginine and leucine with alanine at amino acids 240 and 243 by site-directed mutagenesis. The primers for site-directed mutagenesis were as follows: 5’-TTCTAAGAATGCGATTGTCATGTCTCGGATATTATAACTCCGACAGTCTCTCA-3’; 5’-CTGAGACTGTCCGAGATTATAATATCGCAGGACATGCACCA-

Rad17-APC Cascade in Carcinogenesis

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Cell Culture**—MCF10A, MCF12A, MCF-7, and MDA-MB-231 cells were obtained from the American Type Culture Collection (Manassas, VA). The viral packaging line Phoenix-A cells were the gift from Edward V. Prochownik (University of Pittsburgh). All cells were maintained in DMEM supplemented with 5% or 10% FBS, 1 mmol/liter glucose, and amino acids 240 and 243 by site-directed mutagenesis.

**Plasmids and Constructs**—Rad17DB* (D box mutated Rad17) and Rad17DB** were generated by fusing into Gateway pENTR vector (Invitrogen) and followed by recombination with lentiviral vector pLent6/V5 (Invitrogen). Lentivirus-expressing shRNA specific for Rad17 were obtained from Open Biosystems.

**Antibodies and Tissue Specimens**—Specific antibodies against Rad17 (H-300, Rad17 (H-3), CHK1 (G-4), p-Chk1 (Ser 345), HA-probe (F-7) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against β-actin were from Sigma-Aldrich. Mouse monoclonal antibody against Cdh1 Ab-1 was from Lab Vision (Fremont, CA). Rabbit polyclonal antibody against clasin was from Bethyl Laboratories (Montgomery, TX). Goat anti-rabbit and goat anti-mouse HRP-conjugated secondary antibodies were from Promega (Madison, WI). Tissue microarrays BRC1021 was from Panomics (Richmond, CA).

**Packaging of Virus and Infection of Human**—Packaging of lentivirus was performed in 293T cells using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s instructions. Briefly, 1 day before transfection, the culture dish was replaced with fresh medium without antibiotics. Culture dish was replaced with IMDM without any antibiotics when the cultured cells reach to 90% confluence. 60 μl of Lipofectamine™ 2000 was mixed in 1.5 ml of OPTI-MEM at 25 °C for 5 min. Then, 24 μg of DNA was mixed with helper plasmids RSV-REV, pMDLg/pRRE, and VSV-G in another 1.5 ml of OPTI-MEM. The DNA and Lipofectamine™ 2000 mixture was combined at 25 °C for 25–45 min. The transfection mixture was poured onto the culture dish. The transfection was incubated at 37 °C for at least 8 h or overnight before the medium was changed to 8 ml of complete IMDM with antibiotics. After incubation at 32 °C with 5% CO2 for overnight, the viral supernatant was harvested with a 10-ml syringe and needle. The viral supernatant was filtered with a 0.45-μm filter and further mixed virus with fresh DMEM complete medium (4:1 ratio). The viral mixture was added to a culture plate for infection.

**Co-immunoprecipitation**—Cell pellets collected at designated time points were lysed in buffer (25 mm Tris-Cl, pH 7.5, 150 mm NaCl, 0.5% Nonidet P-40, 1 mm EDTA, 5 mm NaF, and 1× protein inhibitor mixture) on ice for 30 min. Then, 27-gauge one-half-inch syringes were used to shred the DNA. The supernatants were collected after centrifugation at 12,000 × g for 30 min. Equal amount of protein lysates at designated time points were aliquoted, and equal amount of primary antibody was added to the above lysates. After rotation at 4 °C overnight, equal amounts of immobilized protein A/G beads (Pierce, Rockford, IL) were added to the tubes. After rotation again at 4 °C for 4 h, the beads were collected by centrifugation at 2500 × g for 3 min. Electrophoresis loading buffer was added to the beads after washing with IP wash buffer (25 mm Tris-Cl, pH 7.5, 150 mm NaCl, and 1× protein inhibitor mixture) five times. After denaturing at 95 °C for 5 min, the supernatants were subject to Western blot.

**Immunohistochemical Staining**—Tissue sections were dewaxed with xylene and rehydrated through gradient ethanol into water. For antigen retrieval, sections were heated in citrate buffer (pH 6.0) for 10 min at 95 °C in a microwave oven. After cooling to room temperature, the sections were then digested.
with 0.05% trypsin for 10 min at 37 °C. Endogenous peroxidase activity was quenched with 0.3% H2O2 in methanol for 30 min at room temperature. After PBS washes, nonspecific antibody binding was blocked by preincubating slides with 10% normal goat non-immune serum at 37° for 30 min. After blotting off the blocking serum, sections were incubated with primary antibody against Rad17 (1:400 dilution) as well as primary antibody against Cdh1 (1:200 dilution) at 4° overnight. After PBS washes again, sections were incubated with biotinylated secondary antibody at 1:200 dilution for 30 min at room temperature. After incubating with Vectastain ABC reagent (Vector Laboratories, Inc., Burlingame, CA) for 30 min at room temperature, the sections were developed with diaminobenzidine (Sigma-Aldrich). Sections were washed in running tap water and lightly counterstained with hematoxylin, followed by dehydration and coverslip mounting. Negative controls were obtained by omitting the primary antibody. Expression Rad17 and Cdh1 were evaluated as described previously (27). The percentage of positive tumor cells was determined semi-quantitatively by assessing the entire tumor section. Each sample was assigned to one of the following categories: 0 (0–4%), 1 (5–24%), 2 (25–49%), 3 (50–74%), or 4 (75–100%). The intensity of immunostaining was determined as 0 (negative), 1+ (weak), 2+ (moderate), or 3+ (strong). A final immunoreactive score between 0 and 12 was calculated by multiplying the percentage of positive cells with the staining intensity score. All slides were blind evaluated for immunostaining without any knowledge of the clinical outcome of other clinical or pathological data.

**Soft Agar Colony Formation Assays**—The tumorigenicity of Rad17 stabilization was measured by soft agar colony formation assays in duplicate in three independent experiments. Briefly, 1-ml underlayers of 0.6% agar medium were prepared in 35-mm dishes by combining equal volumes of 1.2% noble agar and 2× DMEM with 40% fetal bovine serum (Difco). The cells were trypsinized, centrifuged, and resuspended, and 4 × 10³ MCF7 or 1 × 10⁴ MCF10A cells were plated in 0.3% agar medium. 1-ml toplayers of 0.6% agar medium were prepared and add. The surface was kept wet by addition of a small amount of growth medium. After 2 to 3 weeks, dishes were stained with 0.005% crystal violet and colonies were photographed and counted.

**Clonogenic Assay**—All drugs used in the clonogenic assays were purchased from Sigma-Aldrich Canada, Ltd., and the methods for this assay have been described previously (28). Briefly, cell were plated for 24 h, then culture medium was replaced with either complete medium (for nontreated controls) or complete medium containing one of the following chemotherapeutic agents: cisplatin, doxorubicin, etoposide, methyl methanesulfonate, 5-fluoropyrimidines, mitomycin, taxol, and hydourae for 1 h at concentrations indicated in the figure legends. Cells were then washed once in PBS and replaced with fresh medium. After an additional 7 to 10 days of culture, cells were fixed with an acetic acid/methanol (1:3) solution and stained with a dilute crystal violet (0.33%, w/v) solution, and surviving colonies consisting of 50 or more cells were counted.

**Statistical Analysis**—Statistical analysis was performed using the SPSS statistical software (SPSS, Inc., Chicago, IL). Chi-square test was performed for comparison unless particular test was notified. The results were presented as means ± S.D. p < 0.05 was considered statistically significant.

**RESULTS**

**Aberrant Accumulation of Rad17 Is Detected in Various Breast Cancer Cell Lines and Breast Cancer Tissue**—Previous works have demonstrated that Rad17 is a critical protein governing either activation of DNA damage checkpoint function or termination of checkpoint signaling after the completion of DNA damage response (3–5). Pathological studies using clinical specimens from different stages of human tumors have indicated that loss of genomic integrity due to defective ATR/ATM-DNA damage response network contributes to various tumor initiation (1). Recent studies have revealed that Rad17-mediated DNA damage checkpoint response is tightly regulated by post-translational modification such as phosphorylation and ubiquitin-dependent proteolysis (3–5, 17). Given the aberrant accumulation of Rad17 was measured in various types of cancer cell lines (6–8), several critical questions were raised. 1) Is the accumulation of Rad17 due to abrogation of ubiquitin-mediated protein turnover regulation? 2) If so, does loss of proteolytic regulation of Rad17 contribute to early tumorigenesis or disrupt cellular response to genotoxic stress? To date, we have chosen cultured breast cancer cells as a model to study the impact of Rad17 and its regulation in DNA damage response and breast tumor initiation. We initially measured the Rad17 protein expression in various breast cancer cell lines as well as different type of human breast cancer tissue in comparison with the adjacent normal breast tissue. As shown in Fig. 1, A and B, the Rad17 protein abundance is significantly accumulated in breast cancer cells such as MDA-MB-231, MDA-MB-468, MCF7, T47D, SKBR3, and BT474. Although Rad17 expression levels in normal breast epithelial cell such as MCF10A and MCF12A are low, results from immunohistochemistry analysis indicate that the Rad17 protein levels in the cancer status are much higher than that of in the adjacent normal breast tissue (Fig. 1, C and D). Our immunohistochemistry tissue array further illustrated the higher expression of Rad17 in triple negative type breast cancer than that of in HER2+ and estrogen receptor+/progesterone receptor+ types of breast cancer tissue (Fig. 1E), which is confirmed by analysis of expression and clinicopathological features of breast cancer (Fig. 1F). Taken together, our results demonstrate the pathological connection between aberrant accumulation of Rad17 and breast cancer development.

**Rad17 Protein Turnover Rate Is Significantly Faster in Breast Epithelial Cells Than in Breast Cancer Cell Lines**—Previous studies demonstrated severe accumulated Rad17 in various types of cancer cells (6, 8, 29). We further observed that Rad17 protein fluctuated during the cell cycle, although its role in cell cycle control remains to be addressed (Fig. 2A). The different Rad17 protein expression levels in breast cancer cells in comparison with breast epithelial cells lets us study whether the accumulation of Rad17 in the cancer status is due to the difference in Rad17 protein turnover (Fig. 1, A–D). Thus, we measured the Rad17 in breast cancer cells (MCF7 and MDA-MB-231) and breast epithelial cells (MCF10A and MCF12A). As
shown in Fig. 2, B–E, we observed that Rad17 protein turnover rate in breast epithelial cells MCF10A and MCF12A is much faster than in breast cancer cells MCF7 and MDA-MB-231. Yet, treatment of cells with cycloheximide showed that Rad17 protein half-life in breast epithelial cells is ∼1–2 h (Fig. 2, B and C), whereas it is more than 4 h in breast cancer cells (Fig. 2, D and
FIGURE 2. Rad17 protein turnover rate is significantly faster in breast epithelial cells than that of in breast cancer cell lines. A, fluctuation of Rad17 protein levels during cycle progression, indicating the significant Rad17 accumulation in S phase but low expression during the mitosis. B, short Rad17 protein half-life was measured in MCF10A, a breast epithelial cell line. C, short Rad17 protein half-life was measured in MCF12A, another breast epithelial cell line. D, Rad17 protein half-life (~4 h) was measured in MCF7, an estrogen receptor-positive breast cancer cell line. E, an extended Rad17 half-life was observed in MDA-MB-231, a triple negative breast cancer cell line. CHX, cycloheximide.
Results from the experiments of dosage as well as time course indicated dosage of cisplatin and doxorubicin above 5 μM induced significant Rad17 protein degradation (Fig. 4, A and B), whereas Rad17 dramatically dropped in response to either cisplatin or doxorubicin (5 μM) 4 h after cellular exposure to drug treatment (Fig. 4, C and D). Furthermore, addition of either cisplatin or doxorubicin significantly enhanced the basal turnover of Rad17 in both breast epithelial and breast cancer cells (Fig. 4, E–H, and Fig. 2).

**Stabilization of Rad17 Interferes with DNA Damage Response in Breast Cancer Cells**—Previous studies suggested that Rad17 is involved in several events in DNA damage checkpoint control, which include the following: 1) loading checkpoint complex 9-1-1 to the DNA damage lesion; 2) presenting claspin, which is the critical mediator bridging Chk1 to ATR for phosphorylation/activation; and 3) terminating DNA damage checkpoint signaling for cellular recovery from the genotoxic stress (3–5, 32). To examine the impact of Rad17 in DNA damage response in breast cancer cells, we have engineered a Rad17 degradation-resistant mutant (stable mutant) by mutating destruction boxes on Rad17 (Fig. 5A), and then we measured the response to a series of critical DNA damage proteins as well as cell cycle kinase under Rad17 knockdown or Rad17 stabilization. As shown in Fig. 5, B and C, although depletion of Rad17 significantly blocked the DNA damage checkpoint response (failure to activate Chk1 and arrest cell cycle progression), stabilization of Rad17 significantly prolonged Chk1 activation status that could disrupt cell recovery from genotoxic stress and eventually induce cell death or genome instability. When Rad17 is stabilized, it maintains extended tight interaction with other DNA damage responsive proteins such as claspin, which is a possible reason to maintain unexpected and prolonged Chk1 phosphorylation (Fig. 5D).

**Stabilization of Rad17 Enhances Transformation of Breast Epithelial Cell and Affects Sensitization of Breast Cancer Cells to Chemotherapeutic Drugs**—Demonstration of molecular feature Rad17 proteolytic regulation in steady state status and under the circumstance of DNA damage in breast epithelial as well breast cancer cell implicates the potential impact for Rad17 in regulating genome stability, breast carcinogenesis, as well as targeting therapeutics of breast cancer treatment. Thus, we examined the potential role of Rad17 in the transformation of breast epithelial cell and further assessed the reliance of Rad17 proteolytic regulation in chemosensitization of breast cancer cell. As shown in Fig. 6, A–C, overexpression or stabilization of Rad17 promotes breast epithelial transformation, suggesting disrupted regulation of Rad17 could be a critical early event in breast carcinogenesis. Depletion of Rad17 significantly sensitizes various chemotherapeutic drugs in breast cancer cells, whereas overexpression or stabilization of Rad17 shows more drug resistance (Fig. 6, D and E). Altogether, our findings suggest Rad17 plays an important role in maintaining genomic integrity and homeostasis in breast epithelial and breast cancer cells. Targeting components of Rad17 proteolytic regulation could be a valuable strategy in the chemosensitization of breast cancer cell (Fig. 7).
DISCUSSION

The DNA damage checkpoint system is necessary for genomic stability in which otherwise aberrant regulation can predispose cellular tissues to become cancerous. Upon DNA damage induced by environmental factors or intrinsic genotoxic stress, the DNA damage checkpoint network would arrest or slow down cell cycle progression at G1/S or G2/M, thus enabling DNA repair (33, 34). Abrogation in DNA damage checkpoint could result in altered genomic stability, which could lead to tumor initiation (2, 19, 35, 36). Results from various tumor tissue arrays have shown that loss of genomic integrity due to defective ATR/ATM-DNA damage response cascade contributes to early tumorigenesis (1). Malfunction of Rad17, a critical signaling node of DNA damage signaling, has been linked to
FIGURE 4. Rad17 turnover is regulated in response to various chemotherapeutic agents in breast epithelial and breast cancer cells. A, Rad17 protein levels drop in response to various dosage of cisplatin (CDDP) in MCF7 (measured 4 h after treatment). B, Rad17 protein levels drop in response to various dosage of doxorubicin (DOX) in MCF7 (measured 4 h after treatment). C, cisplatin induces drastic Rad17 degradation in MCF7. D, doxorubicin induces drastic Rad17 degradation in MCF7. E, short Rad17 protein half-life was measured in MCF10A in 5 \( \mu \text{M} \) cisplatin. F, short Rad17 protein half-life was measured in MCF12A in 5 \( \mu \text{M} \) cisplatin. G, Rad17 protein half-life (~4 h) was measured in MCF7 in 5 \( \mu \text{M} \) cisplatin. H, an extended Rad17 half-life was observed in MDA-MB-231 in 5 \( \mu \text{M} \) cisplatin.
various cancer statuses. In our present work, we have dissected the role of Rad17 and the importance of its proteolytic regulation in breast cancer development and anti-breast cancer therapeutics. Our results suggest that precise control of Rad17 protein levels through ubiquitin-proteolysis pathway (Cdh1/APC) is important to maintain cellular hemostasis and ensure normal DNA damage checkpoint function. Disrupted Rad17 proteolytic regulation could affect normal cell cycle control as well as genome stability, which partially explains the physiological relevance of unexpected accumulation of Rad17 protein in variety of cancer type. In addition, our analyses of chemotherapeutic drugs further sketched the important feature for Rad17 in chemosensitization. Overall, results from this study unveiled the connection between Rad17 proteolytic regulation and breast cancer development through aspects of DNA damage checkpoint, cell cycle regulation, tumorigenesis, and chemosensitization, which provide novel insight into breast carcinogenesis and anti-breast cancer therapy (Fig. 7).

**Aberration of DNA Damage Checkpoint Proteins Correlates with Tumorigenesis**—Previous works in studying the connection between DNA damage response and carcinogenesis have revealed that DNA damage responsive proteins such as ATM, ATR, MDC1, Chk2, Mer11, and NBS1 are activated or overexpressed in various cancer tissues (1, 37–39). Although many efforts have been made to further explore the mechanism on how dysregulation of DNA damage response directly links to early tumorigenesis, the explanation is uncertain. It is thought oncogenic factors such as CDK1, cyclin E, E2F, and cdc25A could induce DNA damage possibly due to DNA replication stress and other intrinsic DNA damage stress such as free reactive oxygen species. Afterward, the oncogene-induced DNA damage can turn on the DNA damage checkpoint function that could lead to tumor senescence. This explanation suggested a “barrier function” for the activated/elevated DNA damage response proteins during the process of tumorigenesis.

Our previous and present study of Rad17 in DNA damage response and breast cancer carcinogenesis showed that failure of Rad17 proteolytic regulation results in elevated Rad17 levels, which disrupts the cellular recovery from the DNA damage by impairing termination of checkpoint signal after the completion of checkpoint function (3). We suspect that an additional oncogenic factor could trigger cells for transformation after disruption of cellular metabolic recovery from cell cycle arrest. If true, the ambulation of Rad17 due to dysregulated Rad17 proteolytic regulation could be an indirect cause for tumorigenesis, which explains our result in the connection between aberrant Rad17 with breast cancer status as well as aberration of Rad17 in enhancing breast tumorigenesis.

There are additional possibilities for the connection between the disrupted Rad17 proteolytic regulation and tumorigenesis. We demonstrated that Rad17 protein levels fluctuated drastically during the cell cycle with an accumulated peak at the S phase and reduced expression at G2/M as well as G1 phase. The oscillation pattern of Rad17 in cell cycle suggests a potential role in S phase. Our further experiments using hydroxyurea to induce DNA replication stress revealed the importance of Rad17 in DNA damage response induced by DNA replication stress. To examine the impact of stabilization of Rad17 on nor-

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**FIGURE 5. Stabilization of Rad17 interferes DNA damage response in breast cancer cells.** A, knockdown Rad17 interferes DNA damage response by attenuation of checkpoint function. B, generation of Rad17 stable mutant. Similar to most of Cdh1/APC substrates, Rad17 bears destruction box (a recognition sequence (RXXLXXXN) for the ubiquitin ligase/APC (anaphase promoting complex). C, stabilization of Rad17 interferes DNA damage response by disrupting termination of checkpoint signal after the completion of checkpoint function. D, failure of Rad17 degradation causes prolonged interaction between Rad17 and claspin that in turn lead to extended Chk1 phosphorylation. IB, immunoblot; WCL, whole cell lysates.
**FIGURE 6. Stabilization of Rad17 enhances transformation of breast epithelial cell and affects sensitization of breast cancer cell to chemotherapeutic drugs.**

A, generation of stable Rad17 knockdown cell line in MCF7. B, depletion of Rad17 significantly decreases soft agar clone formation in MCF7. C, overexpressed Rad17 wild-type and DB* mutant increase MCF10A soft agar clone formation. D, depletion of Rad17 significantly sensitize breast cancer cell to various chemotherapeutic drugs in MCF7 and MDA-MB-231. Cells were pretreated with 1.0 μM cisplatin, 1.0 μM methyl methanesulfonate (MMS), 1 mM hydrourea, 1.0 μg/ml mitomycin, 1 mM 5-fluoropyrimidines (5-Fu), 0.01 μM taxol, and 1 μM etoposide for 1 h and then cultured for 2 weeks. E, overexpressed Rad17 wild-type and DB* mutant increase cisplatin resistance in MCF7 cells. Cells were pretreated with 1.0 μM CDDP for 1 h and then cultured for 2 weeks.
normal cell cycle progression, we performed an additional experiment (supplemental Fig. 1). In both MCF7 or MCF10A cells, depletion of Rad17 led to increased S phase population, whereas overexpression of Rad17 or stabilized Rad17 resulted in reduced S phase and accumulated G2/M. Interestingly, either depletion of Rad17 or stabilization of Rad17 significantly caused genome instability (accumulated 6N), further suggesting the disrupted Rad17 regulation could trigger the genomic instability probably through the abrogated checkpoint function or DNA repair due to the intrinsic DNA replication stress.

In addition to the role of Rad17 in DNA replication stress and genomic integrity, we speculate more possible mechanism by which disrupted proteolytic regulation of Rad17 is linked to genomic instability that would eventually result in tumorigenesis. Our previous experiments suggested that Rad17 is a critical DNA damage response protein that orchestrates activation of DNA damage checkpoint as well as time-dependent inactivation of checkpoint signal (in response to either UV or DNA replication stress). In fibroblasts, time-dependent Rad17 degradation after the DNA damage checkpoint function enables metabolic cellular recovery from genotoxic stress, whereas disrupted Rad17 regulation (stabilization) leads to a failure of cell cycle recovery (prolonged accumulated G2/M population) (3). At this point, we speculate that due to heterogeneity of cell population, some cells would commit to mitotic catastrophe/cell death after prolonged G2/M arrest because of stabilized Rad17 (failure to inactivate checkpoint signal after the completion of checkpoint function). In contrast, certain cell populations bypass cell death and survive despite genotoxic stress without successful removal of damaged DNA lesions that in turn contribute to tumorigenesis.

Rad17 Favors Tumor Cell Survival, Thus Proteolytic Regulation of Rad17 Could Be a Novel Strategy for Breast Cancer Chemosensitization—Tumor cells often bear with the severe challenge of intrinsic genotoxic stresses such as DNA replication stress, hypoxia stress, and reactive oxygen species that consistently cause serious DNA damage. To survive, the tumor cell requires even more help to maintain its genome stability through DNA damage response and DNA repair. Thus, adaptation of high expression of Rad17 and other DNA damage responsive proteins is required to guard tumor cell genomic integrity that allows the tumor cell to survive. An abundance of Rad17 protein could favor tumor cell survival from the challenge of genotoxic drugs such as cisplatin and doxorubicin. Down-regulation of Rad17 could shake tumor cell genome stability due to impaired DNA damage response, which could sensitize tumor cells for death in the presence of even lower dosage of chemotherapeutic drug tested in our list (Fig. 6D). Given that Rad17 is a turnover protein that is tightly regulated by ubiquitin E3 ligase (Cdh1/APC), our results suggest a new strategy for the chemosensitization of breast cancer cells by manipulating the ubiquitin-proteasome system such as the Cdh1/APC pathway.

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