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Akt Activation Emulates Chk1 Inhibition and Bcl2 Overexpression and Abrogates G2 Cell Cycle Checkpoint by Inhibiting BRCA1 Foci*[S]

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Akt is perhaps the most frequently activated oncoprotein in human cancers. Overriding cell cycle checkpoint in combination with the inhibition of apoptosis are two principal requirements for predisposition to cancer. Here we show that the activation of Akt is sufficient to promote these two principal processes, by inhibiting Chk1 activation with concomitant inhibition of apoptosis. These activities of Akt cannot be recapitulated by the knockdown of Chk1 alone or by overexpression of Bcl2. Rather the combination of Chk1 knockdown and Bcl2 overexpression is required to recapitulate Akt activities. Akt was shown to directly phosphorylate Chk1. However, we found that Chk1 mutants in the Akt phosphorylation sites behave like wild-type Chk1 in mediating G2 arrest, suggesting that the phosphorylation of Chk1 by Akt is either dispensable for Chk1 activity or insufficient by itself to exert an effect on Chk1 activity. Here we report a new mechanism by which Akt affects G2 cell cycle arrest. We show that Akt inhibits BRCA1 function that induces G2 cell cycle arrest. Akt prevents the translocation of BRCA1 to DNA damage foci and, thereby, inhibiting the activation of Chk1 following DNA damage.

The serine/threonine kinase Akt is perhaps the most frequently hyperactivated oncoprotein in human cancer (1, 2). However, the reasons behind this frequent hyperactivation are not fully explored. One of the most established consequences of Akt activation is inhibition of apoptosis. Inhibition of apoptosis is a critical step in the process of tumorigenesis and is a prerequisite for the genetic instability occurring during this process. Genetic instability that increases mutation rate, and that is associated with predisposition to cancer, is usually acquired through the abrogation of cell cycle checkpoints. However, the abrogation of cell cycle checkpoints by itself could elicit cell death as mechanism to eliminate cells with damaged DNA. Therefore, the inhibition of cell death is required in conjunction with deregulated cell cycle checkpoint to increase mutation rate during tumorigenesis. Certain genetic lesions could provide both cell cycle checkpoint abrogation and inhibition of apoptosis. The best example of such genetic lesion is the inactivation of p53 that overcomes cell cycle checkpoints with concomitant prevention of the elicited apoptosis, which could explain the frequent inactivation of p53 in cancer cells (3, 4). Our previous results suggest that, by analogy to p53 inactivation, the frequent activation of Akt in cancer cells could elicit both inhibition of apoptosis and abrogation of a G2 cell cycle checkpoint in a p53-independent manner (5). However, the mechanism by which Akt exerts its effect on the G2 cell cycle checkpoint is largely unknown.

The p53-independent G2 cell cycle checkpoint following genotoxic stress is mediated by the cell cycle checkpoint protein Chk1. Upon DNA damage, the PI3 kinase-like kinases, ATM, ATR, and DNA-PK are activated and phosphorylate histone H2AX. The phosphorylated H2AX (γH2AX) mediates the polyubiquitylation of histones, H2A and H2AX, at the DNA damage sites through the recruitment of the E3 ubiquitin ligase, RNF8, and the E2-conjugating enzyme Ubc13 (6–10). The polyubiquitylation of histones at the sites of DNA damage creates recognition platforms (DNA damage foci) for other protein complexes, including BRCA1, which is required to facilitate the phosphorylation and activation of Chk1 by ATM and ATR. Subsequently, activated Chk1 executes the G2 cell cycle checkpoint by inhibiting the activity of CDK1 (reviewed in Ref. 11).

Chk1 deficiency results in a severe proliferation defect and cell death in embryonic stem cells; and, as a result embryonic lethality in mice (12). Analysis of conditional Chk1-deficient cell line, demonstrated that these cells have defective G2/M DNA damage checkpoint. Even haplodeficiency of Chk1 elicits impaired response to DNA damage (13). Similarly BRCA1 mutations or deficiency inhibits the G2/M cell cycle checkpoint following exposure to ionizing radiation (IR)[2] or to 6-thioguanine (6TG) treatment (14, 15).

Previously, we have shown that activated Akt inhibits a p53-independent G2 cell cycle checkpoint induced by the exposure to IR or 6-thioguanine (5). However, the exact mechanism(s) by which Akt exerts its effect on the G2 cell cycle checkpoint was not fully explored. Chk1 possesses a consensus Akt phosphorylation site, which was shown to be phosphorylated both *in vitro* and *in vivo* by Akt on serine 280 (16, 17). Serine 280 residue is within the regulatory domain, in close proximity to serine 317 and serine 345, whose phosphorylation is required

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[2] The abbreviations used are: IR, ionizing radiation; shRNA, small hairpin RNA; PBS, phosphate-buffered saline; DAPI, 4′,6-diamidino-2-phenylindole; WT, wild type; GFP, green fluorescent protein; KD, knock-down; 6TG, 6-thioguanine.

for Chk1 activation. Phosphorylation by Akt correlates with impairment of Chk1 activation after DNA damage (17). It was also reported that the phosphorylation of Chk1 at serine 280 leads to its monoubiquitination and facilitates its cytoplasmic localization (18). However, here we report that Ser-280 phosphorylation is not sufficient to recapitulate the ability of Akt activation to overcome the G2 cell cycle checkpoint. We show that activated Akt impairs the phosphorylation and activation of Chk1. Furthermore, we found that BRCA1 localization to DNA damage foci was impaired in the presence of activated Akt. Failure of BRCA1 to localize properly to the site of DNA damage could explain the reduced Chk1 activity in the presence of activated Akt. Activated Akt could also inhibit apoptosis, which is induced as a consequence of overcoming the G2 cell cycle checkpoint.

EXPERIMENTAL PROCEDURES

Cell Lines—Human cell lines HCT116, HCT116 p53$^{-/-}$, HEK293, and rat fibroblasts (Rat1a) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. TOV21G were grown in M199/MCDB medium (1:1) with 15% fetal bovine serum. TOV21G DKD cell lines were established using lentiviral expression system. Lentiviruses harbored small hairpin RNAs (shRNAs) targeting expression of Akt1 and Akt2, or LacZ (19). Cells were treated with 50 μg/ml cycloheximide (MP Biomedicals), 2 mM hydroxyurea (Sigma), or 2 μM 6-thioguanine (Sigma), when indicated.

Retroviruses—The pBabe mAkt and SRα mAkt plasmids were described before in Ref. 20. Retroviruses were generated following transient transfection of retroviral vectors in Phoenix packaging cell lines as previously described (21). The pBabePuro Bcl2 plasmid was described in (21). Rat1a cells were infected with retrovirus carrying Bcl2 and 24 h after infection, cells were selected with 1.5 μg/ml of puromycin.

Adenoviruses—Adenovirus expressing the full length BRCA1 and control adenovirus were amplified by ViraQuest Inc. (North Liberty, IA) to final concentration of 1.2×10^{12} pts/ml. Cells were infected with adenovirus for 3–4 h, in the appropriate medium, containing 5% of fetal bovine serum. Medium volume was reduced to increase viral infection.

Lentiviruses—For shRNA-mediated gene knockdown, the shRNAs were cloned into the lentiviral vector pLenti6/BLOCK-iT (Invitrogen, Carlsbad, CA), and lentiviruses encoding these vectors were generated. The lentiviruses were used to infect Rat1a in the presence of polybrene (8 μg/ml) for about 16 h. About 48 h after infection, the cells were selected using blasticidin (20 μg/ml for 3 days). The sequence of the Chk1 shRNA is: Top strand: 5′-CAC CGC ACA AGG GCT TGA CCA ATT ACG AAT AAT TGG TCA AGC CCT TGT GC-3′; Bottom strand: 5′-AAA AGC ACA AGG GCT TGA CCA ATT ACG AAT AAT TGG TCA AGC CCT TGT GC-3′.

Control shRNA was targeted to LacZ. The sequence for LacZ shRNA is: Top strand: 5′-CAC CGC TAC ACA AAT CAG CGA TTT CGA AAA ATC GCT GAT TTG TGT AG-3′; Bottom strand: 5′-AAA ACT ACA CAA ATC AGC GAT TTT TCG AAA TCG CTG ATT TGT GTA GC-3′.

RNAi-mediated Gene Knockdown—HCT116 p53$^{-/-}$ and HCT116 p53$^{-/-}$ mAkt cells were plated in 6-cm plate (200,000 cells per plate) in DMEM with 10% fetal bovine serum. Forty-eight hours after plating, cells were transfected with 50 nM BRCA1 siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Forty-eight hours after transfection, cells were used for experiment. Human BRCA1 ON-TARGET plus SMART pool™ siRNA was purchased from Dharmacon; and the corresponding negative control used was ON-TARGET plus control siRNA, the non-targeting siRNAs (Dharmacon, Lafayette, CO).

Cloning of Chk1 Mutants—Wild-type Chk1 and SA Chk1 mutant were gift from Dr. Emma Shtivelman. SD Chk1 mutant was made using QuikChange II Site-directed Mutagenesis Kit (Stratagene). For SD Chk1 mutant top strand primer is: 5′-AAA AAG GCC CCG AGT CAC TGA CGG TGG TGT GTC AGA GTC TC-3′, and bottom strand primer is 5′-GAG ACT CTG ACA CAC CAC CGT CAG TGA CTC GGG GCC TTT TT-3′.

All three Chk1 constructs were fused in-frame to EGFP gene from pEGFP-C2 vector (BD Biosciences Clontech, Palo Alto, CA). Chk1 mutants harbored HA tag at 3′-end. This fusion was cloned into retroviral pLPCX vector (Clontech, Palo Alto, CA). Retroviruses were used to infect Rat1a fibroblasts. Forty-eight hours after infection, cells were submitted to short selection with puromycin (1 μg/ml). Experiments were performed immediately after.

Flow Cytometry and Cell Cycle Analysis—Cells were resuspended in PBS and fixed by adding dropwise 96% ethanol. Cells were stored overnight at 4 °C. Before analysis, cells were pelleted by centrifugation at 1,000 rpm for 5 min. The cells were then stained with propidium iodide containing RNase. Flow cytometry was performed using Beckman Coulter EPICS Elite ES (Beckman, Hialeh, FL) and analyzed using Multicycle AV (Phoenix Flow Systems, San Diego, CA).

Western Blot Analysis—The cells were plated at low density and allowed to grow for 24 h, before the application of the various experimental conditions. Afterward, the cells were harvested, and cell extracts were made using ice-cold lysis buffer (20 mM Hepes, 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 10 mM sodium pyrophosphate, 100 mM NaF, 5 mM iodoacetic acid, 20 nM okadaic acid, 0.2 mM phenylmethylsulfonyl fluoride and Complete™ protease inhibitor mixture (Roche Diagnostics)). The extracts were run on 6% SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with the following antibodies: anti-phospho Akt S473 (Cell Signaling), anti-Akt (Cell Signaling), anti-Chk1 (Santa Cruz Biotechnology), anti-BCl2 (Santa Cruz Biotechnology), anti-ATR (Santa Cruz Biotechnology), anti-phospho-Chk1 Ser-345 (Cell Signaling), anti-Cdc25A (Neo Markers), anti-actin (Sigma). Quantification of Western blots was done using ImageJ software.

Apoptosis Assay—Cells were plated at low density (~50,000 cells/well of 6-well plates) and allowed to grow overnight, before subjecting them to γ-irradiation. After 12 h, cells were fixed by gently adding 37% formaldehyde (to a final concentration of 12%) into the culture medium. After overnight fixation, cells were stained with DAPI (1 μg/ml for 5 min) (21). Stained cells were visualized using fluorescence microscopy. The total number of cells as well as the number of apoptotic cells was counted in multiple fields for each sample, and the percentage

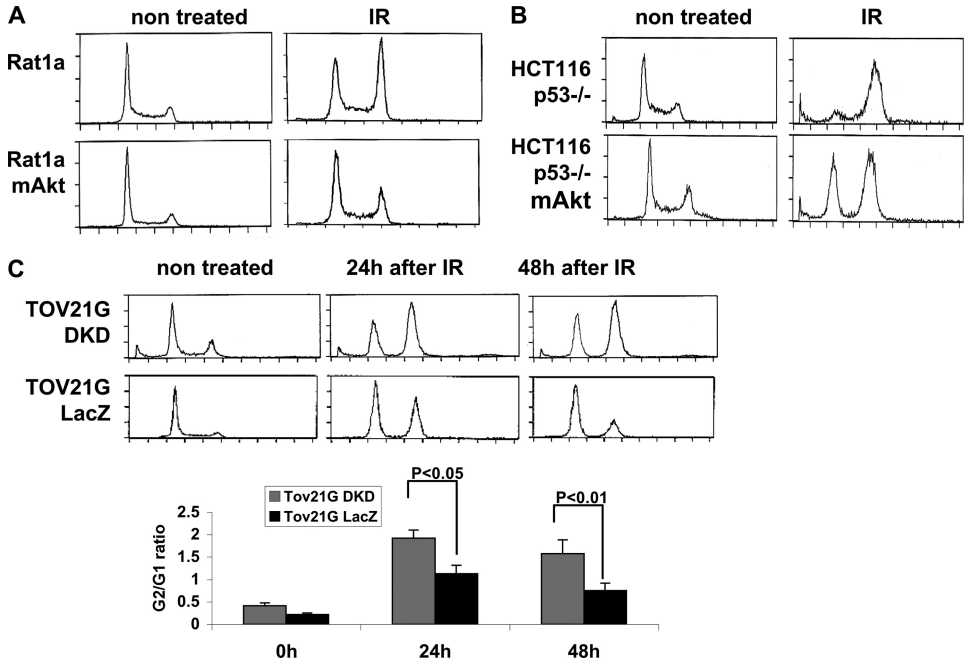

FIGURE 1. **Activation of Akt enables both rodent and human cells to overcome G2 checkpoint after ionizing radiation.** *A*, Rat1a and Rat1a cells expressing activated Akt (*mAkt*) were subjected to ionizing irradiation (10 Gy). Cells were collected for cell cycle analysis 12 h after treatment. *B*, HCT116 p53$^{-/-}$ and HCT116 p53$^{-/-}$ mAkt cells were treated the same as in *A*, except that cells were collected for cell cycle analysis 18 h after treatment. *C*, TOV21G and TOV21G cells in which both Akt1 and Akt2 were knocked down (TOV21G Akt1/2 DKD), and TOV21G shLacZ control cells were irradiated (10 Gy) and subjected to cell cycle analysis 24 and 48 h after treatment. Bar graphs represent G2/G1 ratio as quantified from at least three independent experiments. Data are presented as the mean ± S.E.

of apoptotic cells was calculated. At least 3 different fields, which together contain at least 200 cells, were counted for each sample.

Colony Formation Assay—Cells were plated to very low density (~10,000 cells/10-cm plate). One day following the plating, 6-thioguanine was added to growth medium to a final concentration of 2 μM. Treatment was continued for about 2 weeks, with changing treating medium to fresh one every 3 days. Resulting colonies were rinsed with PBS, fixed with methanol for 3–4 min, and stained with crystal violet. Colonies greater than 1 mm were counted.

Kinase Assay—Chk1 kinase assay was done using the K-Lisa Kit (Calbiotech, San Diego, CA). 1 mg of whole cell lysate was used to immunoprecipitate Chk1 by using anti-Chk1 (Santa Cruz Biotechnology) antibody. Kinase assay and ELISA assay was done following the manufacturer's instruction.

Immunofluorescent Staining—Cells were grown on a four chamber slides. 15,000 cells were plated per chamber day prior to treatment. After appropriate treatment cells were fixed with 4% paraformaldehyde dissolved in PBS. Cells were rinsed with PBS and permeabilized with 0.2% saponin in PBS solution. Cells expressing GFP-fused proteins were mounted after this step using Vectashield with DAPI (Vector Laboratories, Burlingame, CA). Non-GFP expressing cells were rinsed with PBS and blocked for 30 min using 10% FBS and 0.2% Triton X-100 in PBS solution. Slides were blocked for 1 h in humid chamber with anti-BRCA1 antibody (Calbiochem) or anti-γ H2AX antibody in 0.1% saponin, 1% goat serum, PBS solution. Triple wash with blocking solution was performed, and slides were then incubated with fluorescently labeled secondary antibody. After rinsing slides with PBS, they were mounted and sealed. Pictures were taken using Zeiss LSM 5 Pascal Confocal Laser Scanning microscope (Carl Zeiss MicroImaging GmbH, Germany). Digital images were obtained using LSM image software.

RESULTS

Activation of Akt Recapitulates the Combined Effects Mediated by Chk1 Knockdown and Bcl2 Overexpression—To further understand the role of Akt activation on the G2 cell cycle checkpoint, we first repeated our previous results using Rat1a cells. Because of the hypermethylation of p21$^{cip1/waf1}$ promoter (22), the p53-mediated G1 cell cycle checkpoint is impaired in these cells. The cells were exposed to ionizing irradiation (IR) and their cell cycle profile was determined. Rat1a cells accumulated predominantly in the G2 phase of the cell cycle after IR. However, cells expressing activated Akt (Rat1a mAkt cells) (supplemental Fig. S1*A*) maintained the normal cell cycle profile, suggesting that activated Akt enabled cells to overcome G2 arrest (Fig. 1*A*). Similar results were obtained with the human colon carcinoma cells, HCT116, in which p53 was deleted (HCT116 p53$^{-/-}$). HCT116 p53$^{-/-}$ cells responded to IR by a prolonged and robust G2 arrest, which was abrogated and attenuated by activated Akt (Fig. 1*B*). The level and the activity of Akt in these cell is shown in supplemental Fig. S1*B*. These results support the notion that Akt activation can overcome a p53-independent G2 cell cycle checkpoint.

To further establish that Akt controls a G2 cell cycle checkpoint, we used the ovarian cancer cell line, TOV21G, which possesses hyperactivated Akt, even in the absence of serum (19). Upon exposure to IR, these cells were transiently accumulated in G2, but only to a certain extent (Fig. 1*C*). However, the knockdown of both Akt1 and Akt2 in these cells, which markedly reduced total Akt activity ((19) and supplemental Fig. S1*C*), also markedly increased the accumulation of cells in G2 phase of cell cycle for prolonged period. These results provide strong evidence that Akt has a role in controlling the G2 cell cycle checkpoint induced by DNA damage.

When the G2 cell cycle checkpoint is abrogated, the cells may eventually proceed into mitosis but be eliminated by apoptosis or "mitotic catastrophe". As we have shown before (5), activation of Akt not only overcomes the G2 cell cycle checkpoint, but also inhibits the cell death, enabling cells to continue dividing and accumulate mutations. This was manifested by the ability of cells expressing activated Akt to form colonies after a con-

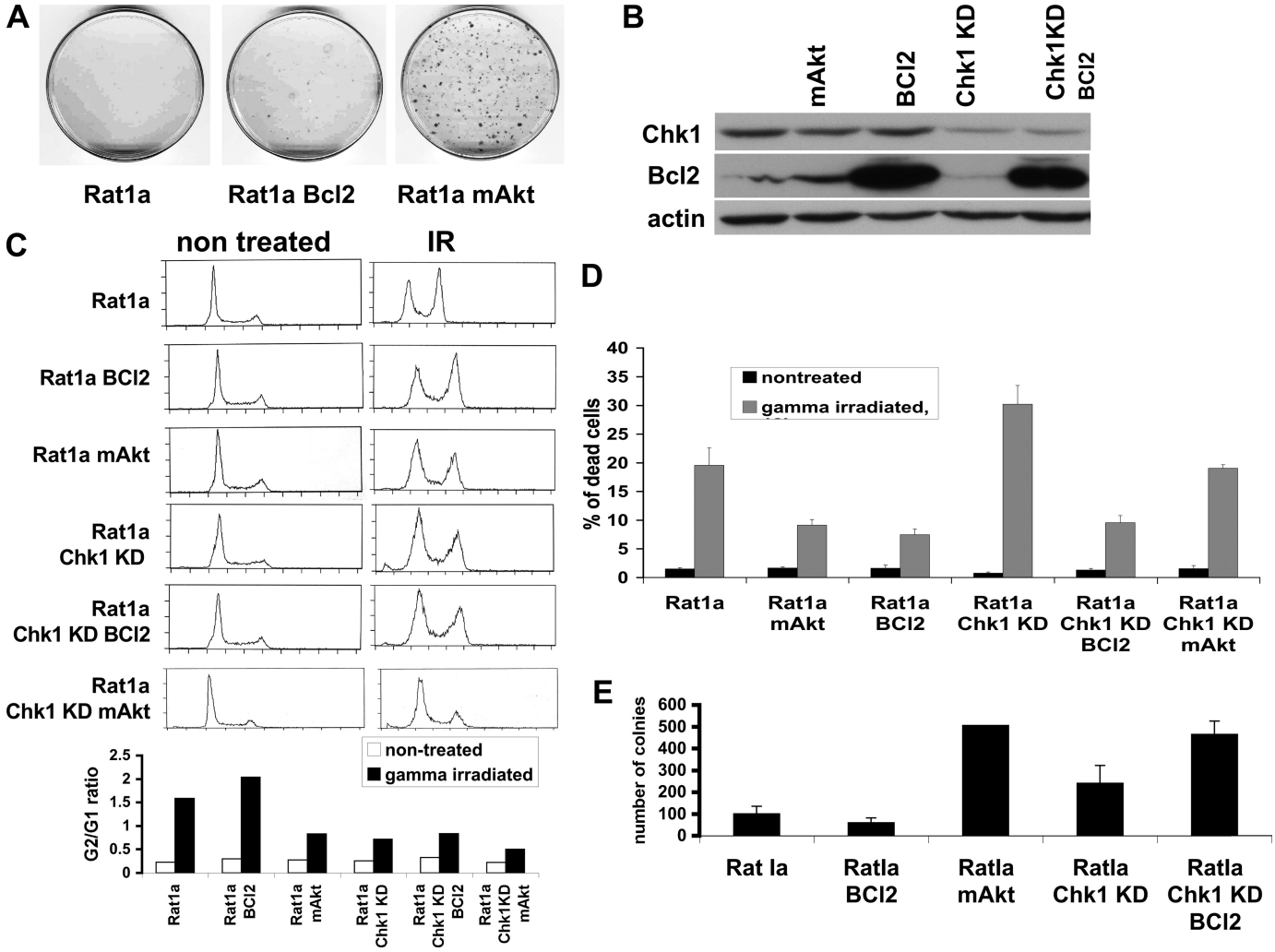

FIGURE 2. **Akt activation recapitulates both inhibition of Chk1 and overexpression of Bcl2.** *A*, Rat1a, Rat1amAkt, Rat1a cells overexpressing Bcl2 (Rat1a Bcl2) were plated at low density and treated with 6-thioguanine, as described under "Experimental Procedures." After prolonged treatment for 2 weeks, cells were fixed and stained with crystal violet. *B*, immunoblot of whole cell lysates from Rat1a, Rat1a mAkt, Rat1a Bcl2, Rat1a Chk1 knock down (Rat1a Chk1KD), and Rat1a Chk1KD cells coexpressing Bcl2. *C*, Rat1a, Rat1a Bcl2, Rat1a mAkt, Rat1a Chk1KD, Rat1a Chk1KD Bcl2, and Rat1a Chk1KD mAkt cells were irradiated and subjected to cell cycle analysis 12 h after irradiation. Bar graphs show the quantification of the flow cytometry results. *D*, cells treated as in *C* were analyzed for apoptosis. Apoptosis was quantified from at least three independent experiments. Data are presented as the mean ± S.E. *E*, bar graph showing number of formed colonies after 6-thioguanine treatment of Rat1a, Rat1a Bcl2, Rat1a mAkt, Rat1a Chk1KD, and Rat1a Chk1KD Bcl2 cells. Quantification of at least three independent experiments done in triplicates is shown. Data are presented as the mean ± S.E.

tinuous exposure to 6TG. 6TG is a guanine analogue, which preferentially pairs with cysteine during replication, and these pairs resemble replication errors and provoke a DNA damage response (23). Consequently, the cells arrest in the G2 phase of cell cycle and eventually undergo cell death.

Rat1a cells are not able to divide and grow in cultured conditions with continuous 6TG presence. The resistance to 6TG can be monitored by the number of colonies that are formed during prolonged treatment. The activation of Akt promotes resistance to prolonged 6TG exposure and enables formation of colonies (Fig. 2*A*). This resistance to 6TG cannot be recapitulated by the overexpression of Bcl2 (Fig. 2*A*), indicating that inhibition of apoptosis by itself is not sufficient to promote the formation of colonies in the presence of 6TG. Being a major effector in regulating G2 checkpoint, Chk1 became a target of our research. We used small hairpin RNA (shRNA) to knockdown Chk1 in the Rat1a cells, which decreased Chk1 protein levels by more than 50% (Fig. 2*B*). Bcl2 was overexpressed in the Chk1 knockdown (Chk1KD) cells. Established stable cell lines overexpressing Bcl2 only, Chk1KD only, or with the combination of Chk1KD and overexpressed Bcl2 (Fig. 2*B*) were exposed to IR.

As shown in Fig. 2*C*, the knockdown of Chk1 overrides the G2 arrest to a similar extent as does activated Akt. Overexpression of Bcl2, by itself did not overcome the G2 arrest, but the combination of both Chk1KD and overexpression of Bcl2 overcame the G2 arrest to the same extent as the Chk1KD alone. Chk1KD in combination with activated Akt further increased the resistance to G2 arrest following IR (Fig. 2*C*). Unlike the cells, expressing activated Akt, the Chk1KD cells, despite overcoming the G2 arrest, displayed a dramatic increase in apoptosis (Fig. 2*D*). However, when Bcl2 was overexpressed in the Chk1KD cells, both the G2 arrest and apoptosis were inhibited, recapitulating Akt activation (Fig. 2, *C* and *D*). We then exposed these cells to 6TG continuously, and quantified colony formation. The results clearly show that, like the activation of Akt, the

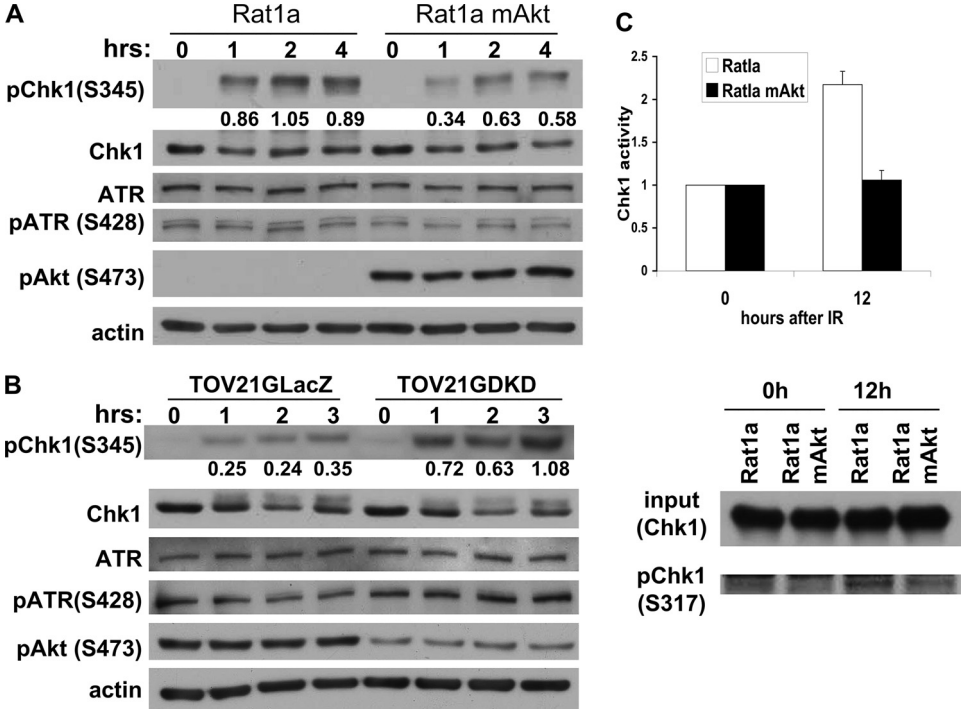

FIGURE 3. **Activated Akt inhibits the activation of Chk1 following DNA damage.** *A*, Rat1a and Rat1a mAkt cells were treated for 0, 1, 2, or 4 h with 2 mM hudroxyurea. Whole cell lysates were analyzed by immunoblotting for p-Chk1, p-ATR, and p-Akt. The ratios of pChk1/Chk1 are indicated. *B*, TOV21G shLacZ and TOV21G Akt1/2 DKD cells were treated for 0, 1, 2, and 3 h with 2 mM hydroxyurea. Whole cell lysates were analyzed by immunoblotting for p-Chk1, p-ATR, and p-Akt. The ratios of pChk1/Chk1 are indicated. *C*, Rat1a and Rat1a mAkt cells were exposed to IR, and the activity of Chk1 was determined using *in vitro* kinase assay as described under "Experimental Procedures." Results are shown relative to the values obtained for the untreated cells. Bar graph represents results of three independent experiments. Data are presented as the mean ± S.E. Immunoblot shows the amount of Chk1 utilized for kinase assay after immunoprecipitation with anti-Chk1 antibodies.

combination of Chk1KD and overexpression of Bcl2 enables cells to divide and survive under continuous exposure to DNA damage (Fig. 2*E*). Taken together, these results clearly demonstrate that the activation of Akt is capable of overcoming Chk1 function and promoting cell division and cell survival under DNA damage. This observation could explain the frequent activation of Akt in human cancers, as it enables both cell proliferation and cell survival despite the accumulation of mutations, which otherwise would prohibit cell proliferation and survival.

Akt Inhibits the Activation of Chk1 following DNA Damage—To investigate the mechanism through which Akt affects Chk1, we first looked at Chk1 level and stability in response to Akt activation. We blocked protein synthesis for up to 8 h by treating cells with the protein synthesis inhibitor cycloheximide, and monitored Chk1 protein levels. As shown in supplemental Fig. S2*A*, the half-life of Chk1 did not change when activated Akt was expressed in the Rat1a cells. To determine if this observation is also true for human cells, the same experiment was performed in the HEK293 cells (supplemental Fig. S2*B*). We compared the half-life of Chk1 in the presence or absence of activated Akt. As with Rat1a cells, activation of Akt did not affect Chk1 stability in HEK293 cells, even when these cells were exposed to a checkpoint-activating reagent, such as hydroxyurea (supplemental Fig. S2*C*). Based on these findings, we concluded that Akt does not affect Chk1 protein stability.

To understand the mechanism by which Akt may affect the function of Chk1, we examined Chk1 activation after checkpoint activation. Rat1a and Rat1a mAkt cells were treated with hydroxyurea (Fig. 3*A*). We collected cells at different time intervals throughout the treatment, and followed Chk1 phosphorylation on serine 345 as readout for its activity. Chk1 phosphorylation on serine 345 was decreased in the cells expressing activated Akt. The level of Chk1 phosphorylation was lower for each time point tested (Fig. 3*A*). Notably, the level of Chk1 kinase, ATR, was not affected by Akt.

We used a loss of function approach to further substantiate the role of Akt in Chk1 phosphorylation/activation. We treated TOV21G cells and TOV21GAkt1/2 DKD cells, in which both Akt1 and Akt2 were knocked down (19), with hydroxyurea, and found that the induction of Chk1 phosphorylation was markedly higher after the knockdown of Akt1 and Akt2 (Fig. 3*B*). Taken together, these results suggest that the activation of Akt interferes with Chk1 activation but does not seem to affect ATR, the kinase that phosphorylates and activates Chk1. To assess directly the effect of Akt activation on Chk1 kinase activity, we performed Chk1 kinase assays. We utilized Rat1a and Rat1a (mAkt) cells. Both cell lines were irradiated and, 12 h later, total cell extracts were collected. Chk1 was immunoprecipitated and subjected to *in vitro* kinase assay (Fig. 3*C*). As expected, Chk1 kinase activity was increased following irradiation of Rat1a cells. However, in Rat1a cells expressing activated Akt, no increase in Chk1 activity was observed (Fig. 3*C*). Collectively, these results strongly suggest that activation of Akt impairs the induction of Chk1 activity in response to DNA damage independently or downstream of ATR.

Direct Phosphorylation of Chk1 by Akt Does Not Phenocopy the Ability of Akt to Overcome G2 Cell Cycle Checkpoint—Because it was previously shown that Akt could directly phosphorylate Chk1 on serine 280 (17), we wanted to determine if this phosphorylation can affect Chk1 function following DNA damage. For this purpose, we have generated Chk1 mutants in which serine 280 was converted to either alanine (loss of phosphorylation) or to aspartate (phosphomimetic). WT Chk1 and Chk1 mutants were fused in-frame with GFP. Retroviruses expressing the WT and Chk1 mutants were generated to infect Rat1a (Chk1KD) cells and to establish stable polyclonal cell lines (Fig. 4*A*). The cells were subjected to IR and analyzed by flow cytometry (Fig. 4*B*). WT and Chk1 mutants were able to restore G2 arrest fully in Chk1KD cells, indicating that Ser-280

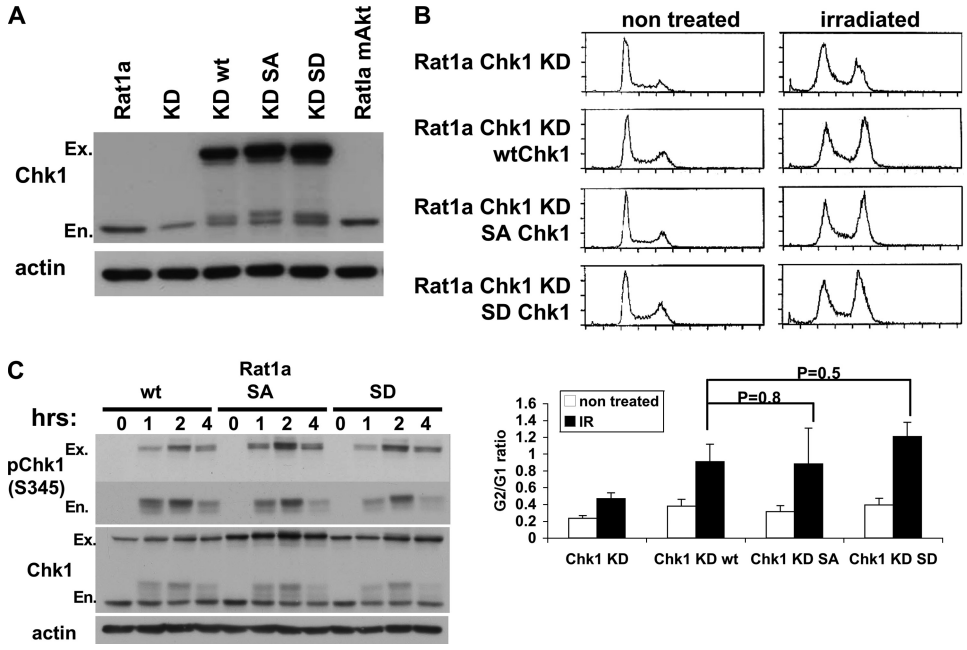

FIGURE 4. **Both WT and Chk1 mutants in the Akt phosphorylation site restore the G2 cell cycle checkpoint in Rat1a Chk1KD cells.** Rat1a Chk1KD cells were infected with high titer retroviruses expressing either WT Chk1, S280A, or S280D mutant fused in-frame with eGFP. *A*, immunoblot showing the expression of both endogenous and exogenous WT and Chk1 mutants in Rat1a Chk1KD cells. *B*, cell cycle analysis of Rat1 Chk1KD, and Rat1a Chk1KD expressing WT or Chk1 mutants cells after IR. Bar graphs showing G2/G1 ratio represent results of three independent experiments. Data are presented as the mean ± S.E. *C*, immunoblot showing the phosphorylation of endogenous Chk1, and exogenous WT or Chk1 mutants following treatment with 2 mM hydroxyurea.

phosphorylation is not sufficient to affect the ability of Chk1 to induce G2 arrest. Furthermore, WT and Chk1 Ser-280 mutants were phosphorylated to a similar extent on Ser-345 in response to hydroxyurea (Fig. 4*C*).

Activation of Akt Inhibits BRCA1 Foci following DNA Damage and Phenocopy BRCA Deficiency—It had been previously shown that BRCA1 activates Chk1 following IR or 6-TG exposure, and that BRCA1 is required for Chk1 phosphorylation and the activation of the G2 cell cycle checkpoint (14, 15). Indeed, when we knocked down BRCA1 in HCT116 $p53^{-/-}$ cells, Chk1 phosphorylation was impaired (Fig. 5*A*), inhibited the G2 arrest following IR (Fig. 5*B*).

Overexpression of BRCA1 is known to cause G2 cell cycle arrest in the cells (24). To determine if Akt activation is able to overcome the G2 arrest induced by BRCA1, we utilized adenovirus carrying full-length human BRCA1 and examined the effect of BRCA1 overexpression on cell cycle. After adenovirus infection, HCT116 p53−/− cells were collected, and their cell cycle profile was analyzed. Overexpression of BRCA1 elicits Chk1 activation, as measured by Ser-345 phosphorylation, but not in cells expressing activated Akt (Fig. 5*D*). Consistently, the control HCT116 $p53^{-/-}$ cells showed increased numbers of cells in G2 after infection with adenovirus carrying BRCA1. However, HCT116 $p53^{-/-}$-expressing mAkt cells displayed a diminished arrest, even though the same dose of virus was used. Similarly, overexpression of BRCA1 induced cell cycle arrest in Rat1a cells but not in Rat1a cell expressing activated Akt or in Chk1KD cells (Fig. 5*E*). These results provide convincing evidence that activation of Akt mimics either BRCA1 or Chk1-deficiency, and raise the possibility that Akt exerts its effect through BRCA1.

To address further the possibility that Akt exerts its effect on G2 cell cycle checkpoint through BRCA1, we first determined if activation of Akt could affect the level or stability of BRCA1 protein. We therefore analyzed the stability of BRCA1 protein in HCT116 $p53^{-/-}$ and HCT116 $p53^{-/-}$ mAkt cells. Cells were treated with cycloheximide to inhibit *de novo* protein synthesis, and BRCA1 protein level was determined by immunoblotting at different time points after treatment. As shown in supplemental Fig. S6, there was no significant difference between control cells and cells expressing activated Akt, indicating that Akt does not affect the level or stability of BRCA1.

BRCA1 exerts its effect on G2 cell cycle checkpoint and Chk1 activity by translocating to DNA damage foci following DNA damage (11). Therefore, we examined whether Akt could affect the translocation of BRCA1 to DNA damage foci. HCT116 $p53^{-/-}$ and HCT116 $p53^{-/-}$ mAkt cells were exposed to IR and after 2 and 4 h, cells were fixed and stained with BRCA1 and γH2AX antibodies. Representative pictures of cells were taken, using confocal microscopy, and co-localization of γH2AX and BRCA1 was quantified (Fig. 6). As expected, we observed BRCA1 localization to DNA foci as early as 2 h after irradiation of HCT116 $p53^{-/-}$ cells, which coincides with γH2AX staining. However, the activation of Akt markedly inhibited the BRCA1 foci. Distribution of BRCA1 in the non-treated cells was identical, regardless of Akt activation. These experiments demonstrated that Akt activation prevents BRCA1 from relocalizing to the foci formed at the sites of DNA damage. Failure of BRCA1 to translocate to the site of DNA damage in the cells with activated Akt could potentially explain the incomplete phosphorylation and activation of Chk1 by Akt activation.

DISCUSSION

The frequency by which Akt is activated in human cancers is perhaps similar or higher then the frequency by which the tumor suppressor p53 is inactivated in human cancers (1, 2). Akt is activated in human cancers by multiple mechanisms, most notably through the loss of PTEN function and through activation mutations in the gene encoding p110, the catalytic subunit of PI3K (25). One major reason for the frequent inactivation of p53 in human cancers is because its loss of function induces predisposition to cancer through impaired cell cycle checkpoint combined with the inhibition of apoptosis. Here we provided evidence that the activation of Akt could also induces abrogation of cell cycle checkpoint combined with inhibition of apoptosis. We showed that activation of Akt, which overcomes

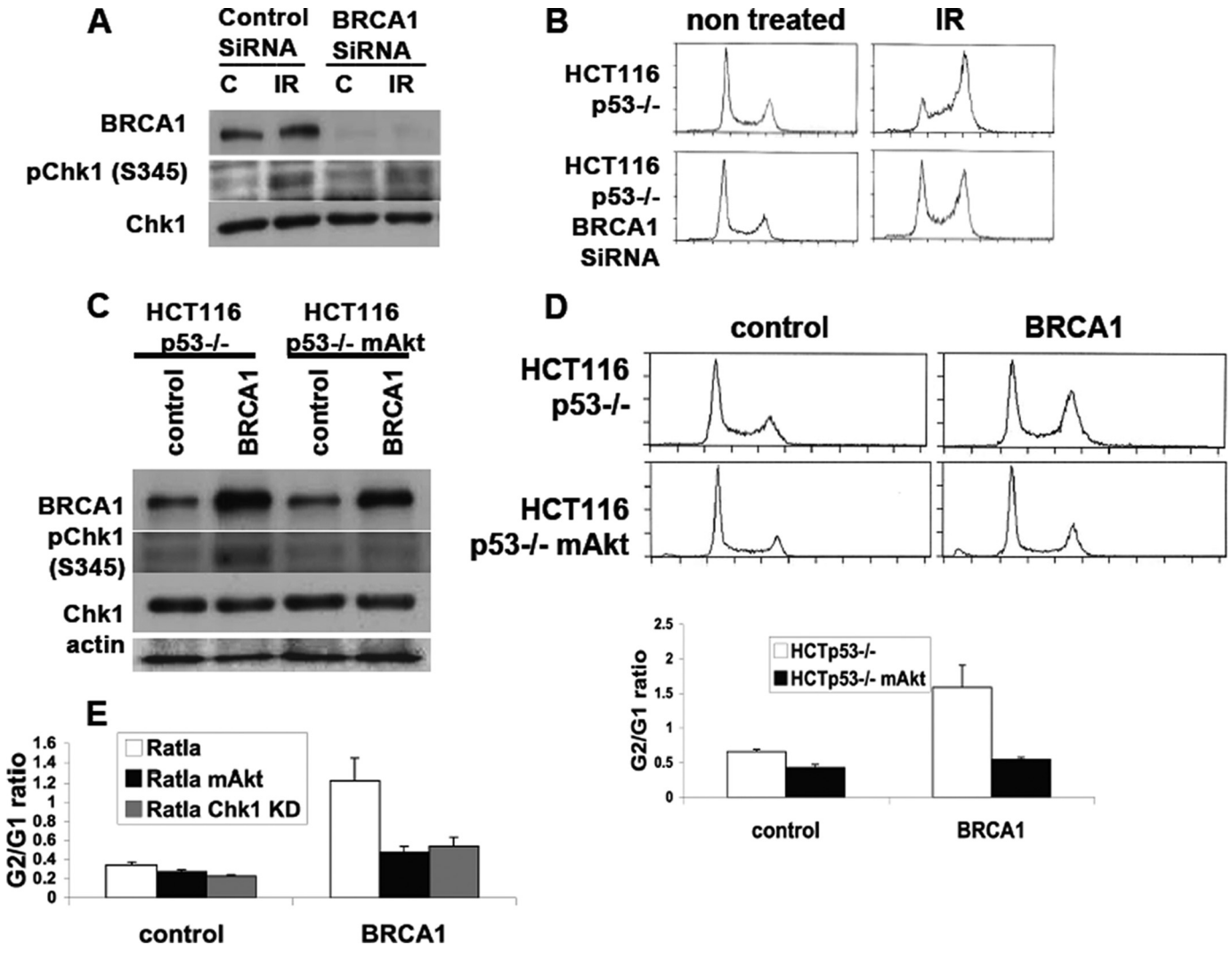

FIGURE 5. **Akt inhibits G2 arrest induced by BRCA1.** *A*, HCT116 p53$^{-/-}$ cells were transfected with either BRCA1 siRNA or control siRNA and cell extracts were subjected to immunoblotting. *B*, HCT116 p53$^{-/-}$ cells transfected with either BRCA1 siRNA or control siRNA were exposed to IR, fixed, and analyzed by flow cytometry. *C*, HCT116 p53$^{-/-}$ and HCT116p53$^{-/-}$ mAkt cells were infected with adenovirus expressing BRCA1. Cell lysates were prepared 48h after infection and subjected to immunoblotting. *D*, cells were fixed 48 h after infection and analyzed by flow cytometry. Bar graph is representing an average G2/G1 ratio of three independent experiments. Data are presented as the mean ± S.E. *E*, Rat1a, Rat1a mAkt, and Rat1a Chk1KD fibroblasts were infected with BRCA1. Twenty-four hours after infection, cells were fixed and analyzed as in *D*.

a p53-indepedent G2 cell cycle checkpoint, promotes cell proliferation in the presence of DNA damage. This activity of Akt cannot be recapitulated by the knockdown of Chk1 only as it requires an inhibitor of apoptosis. Akt activation could provide both processes, which can only be recapitulated by the knockdown of Chk1 in combination with the overexpression of Bcl2.

The ability of activated Akt to overcome a p53-independent cell cycle checkpoint is significant because it is likely that most cancer cells in which Akt is hyperactive, display also a p53 loss of function. This is because for Akt to be activated in cells, the loss of p53 is required in order to prevent premature senescence. Akt activation was shown to induce a p53-dependent premature senescence (19, 26), and therefore, the onset of tumors induced by the loss of PTEN is markedly accelerated by the deletion of p53 (26). Thus, the combination of Akt activation and the loss of p53 function could provide a complete resistance to DNA damage-induced cell cycle checkpoints.

Because the knockdown of Chk1 inhibits the G2 cell cycle arrest to a similar extent as does activated Akt, and because Akt was shown to phosphorylate Chk1, we first focused on the possibility that Akt can directly modify Chk1 function. Surprisingly, however, we found that the phosphorylation of Chk1 by Akt is insufficient to phenocopy the ability of Akt to overcome the G2 cell cycle arrest. Because we could not find any significant effect of Akt on the Chk1 kinase, ATR, we examined the possibility that Akt affects BRCA1, acting upstream of ATR and Chk1. BRCA1 was shown to be required for Chk1 activation following DNA damage by translocating to DNA damage foci, and presumably facilitating phosphorylation of Chk1 by ATR or ATM. It was also shown that, like the activation of Akt, BRCA1 is required for both IR- and 6TG-induced G2 cell cycle checkpoint (14, 15). Indeed, we found that activated Akt could override the G2 cell cycle arrest induced by BRCA1, and that the knockdown of BRCA1 inhibits IR-induced G2 cell cycle arrest with concomitant reduction in Chk1 activation, and to a similar extent as does activated Akt. Mechanistically, we found that activated Akt inhibits the localization of BRCA1 to DNA damage foci. Thus, we concluded that Akt inhibits Chk1 activ-

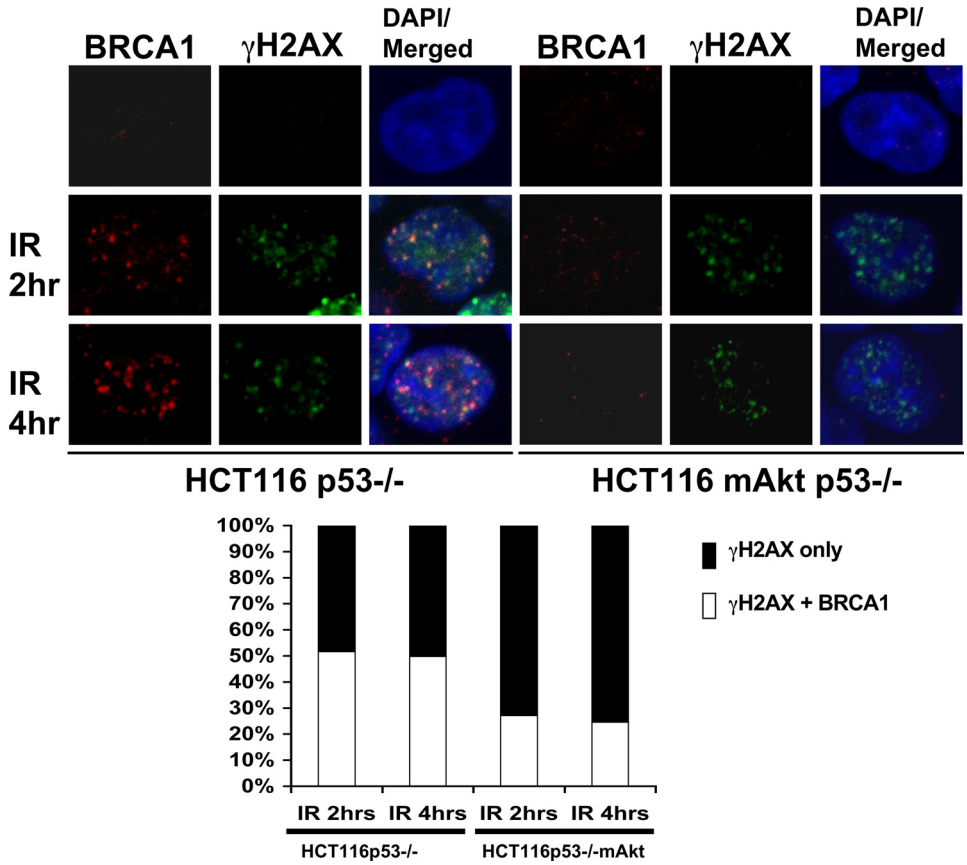

FIGURE 6. **Activated Akt inhibits BRCA1 targeting to DNA damage induced foci.** HCT116 $p53^{-/-}$ and HCT116 $p53^{-/-}$ mAkt cells were treated with ionizing radiation of 10 Gy. 2 and 4 h after treatment, cells were fixed and immunostained with anti-BRCA1 and anti-γH2AX, counterstained with DAPI, and detected by immunofluorescence. About 200 foci in 20 random nuclei per each line and per each treatment were monitored for γH2AX staining only and co-staining for γH2AX and BRCA1. Bar graphs present the percentage of foci with γH2AX staining only, and co-staining of γH2AX and BRCA1.

ity by inhibiting the ability of BRCA1 to localize to DNA-damage foci and to facilitate the phosphorylation and activation of Chk1 by ATM and ATR. Although additional work is required to delineate the exact mechanism by which Akt exerts its effect on BRCA1, there are several possibilities to consider. One possibility is that Akt affects BRCA1 by direct phosphorylation as it was shown that Akt can phosphorylate threonine 509 of BRCA1 *in vitro* (27). Interestingly, this residue is located in the one of the two nuclear localization sequences (NLS) of BRCA1 protein. Even though we did not see any obvious difference in the distribution of BRCA1 in the untreated cells, the activation of Akt clearly inhibited localization of BRCA1 to the DNA damage foci after ionizing radiation. One potential scenario could be that phosphorylation of threonine 509 prevents proper protein-protein interaction between BRCA1 and other proteins found in the DNA damage foci. The candidate to test would be RAP80. Published reports have identified several distinct complexes, which rely on the protein RAP80 to form radiation-induced foci of BRCA1 (28–30). BRCA1 interacts through BRCT domain with RAP80 (29). Akt phosphorylation of BRCA1 could interfere with this interaction and thus prevent recruitment of BRCA1 to DNA damage foci. More recently, it was shown that MERIT40/NBA1 is required for the recruitment of BRCA1 to the DNA damage foci (31–34), and for the integrity of BRCA1-RAP80 complex (32). Interestingly, MERIT40 possesses an Akt phosphorylation site that overlaps with 14-3-3 binding site, raising the possibility that Akt phosphorylation could induce sequestration of MERIT40 by 14-3-3.

Previously published work had shown that Chk1 phosphorylation by Akt on serine 280, interferes with the nuclear localization of Chk1 (18). We confirmed that activated Akt decreases nuclear localization of Chk, both in the absence and presence of DNA damage (supplemental Fig. S3). However, as discussed above, we found that serine 280 phosphomimetic mutant of Chk1 could not recapitulate, Akt activity. Furthermore, we found that although Chk1KD overcomes the G2 arrest induced by BRCA1 (Fig. 5*E*), expression of serine 280 phosphomimetic mutant of Chk1 in the Chk1KD cells cannot overcome the G2 arrest induced by BRCA1 (supplemental Fig. S4). Nevertheless, we cannot not completely rule out the possibility that the phosphorylation of Chk1 by Akt contributes to the abrogation of the G2 cell cycle checkpoint in conjunction with the impaired BRCA1 localization to DNA damage foci. Finally, it was reported that BRCA1 can reduce the level of phosphorylated Akt (35). We did not find that the knockdown of BRCA1 affected the level of phosphorylated Akt (supplemental Fig. S5), and therefore it cannot explain our results. However, we cannot completely exclude the possibility that reduced Akt activity by BRCA1 was instated as a feedback negative mechanism that restrains Akt activity and attenuates the effect of Akt on the activity of BRCA1.

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