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BRCA1 Interacts with and Is Required for Paclitaxel-Induced Activation of Mitogen-Activated Protein Kinase Kinase Kinase 3

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ABSTRACT

BRCA1 has been implicated in a number of cellular processes, including transcriptional regulation, DNA damage repair, cell cycle arrest, and apoptosis. We identified mitogen-activated protein kinase (MAPK) kinase kinase 3 (MEKK3), an upstream regulator of the c-Jun NH_2-terminal kinase/stress-activated protein kinase and p38/MAPK pathways, as a novel BRCA1-interacting protein in a yeast two-hybrid screen and confirmed the interaction by coimmunoprecipitation in mammalian cells. Deletion mapping demonstrated that amino acids 1611–1863 are required to mediate the interaction with MEKK3 in yeast. BRCA1 disease-associated mutations abrogated the interaction in yeast, and BRCA1 failed to interact with MEKK3 in BRCA1 mutant HCC1937 breast cancer cells. We demonstrate that small interfering RNA-based inhibition of endogenous BRCA1 reduces MEKK3 kinase activity and conversely that inducible expression of BRCA1 activates MEKK3 and p38/MAPK. Finally, we demonstrate using complementary approaches that BRCA1 is required for paclitaxel-induced activation of MEKK3. These data indicate that BRCA1 is a key regulator of the paclitaxel-induced stress response pathway and suggest that the ability of BRCA1 to associate with, and mediate the activation of, MEKK3 represents a potential mechanism through which this pathway is regulated.

INTRODUCTION

BRCA1 encodes a tumor suppressor gene that is mutated in the germline of women with a genetic predisposition to breast and ovarian cancer (1). Germline mutations of BRCA1 are found in approximately 50% of breast-ovarian cancer pedigrees and in approximately 10% of women with early-onset breast cancer, irrespective of family history (2). Somatic inactivation of BRCA1 is rare in sporadic breast cancers (3); however, approximately one-third of sporadic tumors exhibit reduced expression of BRCA1 protein, indicating that epigenetic mechanisms also may play a role in regulating BRCA1 expression (4, 5). Although the exact function of BRCA1 remains to be defined, roles in DNA damage repair, transcriptional regulation, cell cycle checkpoint control, and ubiquitination have been inferred (6).

Substantial evidence exists to suggest that BRCA1 plays a critical role in modulating the cellular response to stress and in particular to agents that cause DNA damage.

BRCA1 becomes hyperphosphorylated in response to various DNA-damaging agents, including γ-irradiation, an effect that is mediated in part by the ataxia telangiectasia mutated (ATM; Ref. 7) and CHK2 kinases (8). BRCA1 has been shown to colocalize at sites of DNA damage with RAD51, the human homologue of bacterial RecA, which is involved in homologous recombination repair following ionizing radiation (9). Furthermore, BRCA1 is a component of the RAD50, Mre11, and NBS1 complex implicated in homologous recombination and nonhomologous end joining (10). It more recently has been reported that BRCA1 resides within a large DNA repair protein complex called BRCA1-associated genome surveillance complex that includes various mismatch repair proteins, including MLH1, MSH2, and MSH6, suggesting a role for BRCA1 in mismatch repair (11). Together, these reports suggest that BRCA1 is a component of multiple repair pathways, which remain to be fully investigated. It also has been suggested that BRCA1 functions as a sensor of DNA damage relaying signals to the cell cycle checkpoint and cell death machinery. A number of studies have correlated BRCA1 deficiency with aberrations in cell cycle checkpoint control. Human tumor cells lacking functional BRCA1 demonstrate a high frequency of chromosome aneuploidy, characteristic of a defective G_2-M checkpoint (12). It also has been demonstrated that BRCA1 is required for S-phase and G_2 arrest following ionizing irradiation, an effect that is dependent on differential phosphorylation by ATM (13, 14). Furthermore, genetic instability has been observed in BRCA1 exon 11 isoform-deficient mouse embryonic fibroblasts, resulting from a defective G_2-M checkpoint and centrosome amplification (15). We previously have demonstrated that BRCA1 can activate the G_2-M and spindle checkpoints in response to agents that disrupt the mitotic spindle, such as paclitaxel, suggesting that BRCA1 plays a more general role in the regulation of stress-response pathways (16).

A number of studies also have implicated BRCA1 in the regulation of apoptosis following cellular stress. We initially demonstrated that exogenous expression of BRCA1 induced apoptosis, an effect that was dependent on c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) activation (17). It subsequently was reported that BRCA1 modulates stress-induced apoptosis through a pathway that involves the JNK, Fas, and Fas ligand and the activation of caspase-9 (18). In contrast, BRCA1-deficient cells exhibit a radiosensitive phenotype following exposure to a range of DNA-damaging agents, including ionizing radiation (8), and the DNA cross-linking agents cisplatin and mitomycin C (19, 20). This radiosensitive phenotype, which can be reversed following exogenous expression of wild-type BRCA1, has been associated with a failure in BRCA1-dependent DNA damage repair pathways. Consistent with this, we have reported that reconstitution of BRCA1 in the BRCA1-deficient HCC1937 cell line induces resistance to a range of DNA-damaging agents, including etoposide, bleomycin, and cisplatin. Furthermore, we demonstrated that the resistant phenotype is caused by BRCA1-dependent inhibition of apoptosis (21). In contrast, however, we also have demonstrated that BRCA1 dramatically sensitizes breast cancer cells to apoptosis induced by agents that disrupt the mitotic spindle, including vinorelbine and paclitaxel (21). Similarly, we have reported that BRCA1 dramatically sensitizes breast cancer cell lines to IFN-γ-mediated apoptosis, indicating that BRCA1 may regulate apoptosis in response to diverse stress signals (22). Therefore, it appears that BRCA1 can

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Note: P. M. Gilmore and N. McCabe contributed equally to this work.

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regulate proapoptotic and antiapoptotic pathways, depending on the nature of the cellular stress.

The mechanism through which BRCA1 modulates such diverse signaling pathways remains a matter of speculation; however, it is likely to relate to its role as a substrate for numerous stress response kinases and its ability to regulate the expression of key downstream target genes. It also is becoming increasingly clear that not only does BRCA1 represent a target of stress response kinases but also that it functions to regulate kinase activity. It has been reported that BRCA1 can associate with and mediate the activation of CHK1 following γ-irradiation resulting in G_2-M arrest, although the mechanism through which this is achieved was unclear (23). Similarly, BRCA1 has been reported to mediate ATM- and ataxia telangiectasia mutated and Rad3 related (ATR)-dependent phosphorylation of p53, c-Jun, Nbs1, and CHK2 but not the DNA-associated substrates Rad9, Hus1, and Rad17 (24). This has led to the suggestion that BRCA1 is required specifically for the phosphorylation of ATM and ATR targets involved in cell cycle arrest and apoptosis (24). In the current study, we have identified mitogen-activated protein kinase (MAPK) kinase kinase 3 (MEKK3), an upstream regulator of the JNK and p38 MAPK pathways, as a BRCA1-interacting protein in a yeast two-hybrid screen using the BRCT motif as bait. We present evidence to suggest that the interaction between BRCA1 and MEKK3 is functionally important and demonstrate that BRCA1 is required for paclitaxel-induced activation of MEKK3.

MATERIALS AND METHODS

Cell Lines. The BRCA1-inducible cell line MBR62-bcl2 was generated from the MDA435 breast cancer cell line and maintained as described previously (16). MDA435 cells were cultured in DMEM supplemented with 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μg/ml penicillin/streptomycin. MCF-7 cells were cultured in DMEM supplemented with 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μg/ml penicillin/streptomycin. HCC1937 cells were cultured in RPMI supplemented with 20% FCS, 2 mM L-glutamine, and 50 μg/ml penicillin/streptomycin. HCC-EV and HCC-BRCA1 cells were generated by transfecting the BRCA1 mutant HCC1937 cells with either empty Rc/CMV or Rc/CMV-BRCA1, followed by selection in 200 μg/ml geneticin as described previously (21).

Plasmid Construction. The pDBleu-BRCA1 plasmid was generated by amplifying bases 4603–5711 (encoding amino acids 1475–1863) of BRCA1 from pUHD-10^{-3}-BRCA1 using the forward primer 5′-agtccagtcagcc aggtcatccccttcaaaatgcc-3′ and the reverse primer 5′-gagcgcgcggccgcgt agtggctgtgggggatctgg-3′ (17). *Sal*I/*Not*I restriction sites were included on the forward and reverse primers, respectively, to facilitate cloning into the pDBleu vector. Mutant bait constructs were generated from pDBleu-BRCA1 by site-directed mutagenesis. Mutation 1 introduced a C→A missense mutation at nucleotide 5242, resulting in an amino acid change from alanine to glutamine, and was generated using the primer pair 5′-ctaggaattg*a*gggaggaaaatggg-3′ and 5′-ccaattttcctccc*t*caattcctag-3′. Mutation 2 introduced a T→G missense mutation at nucleotide 5443, resulting in an amino acid change of methionine to arginine using the primer pair 5′-ccttcaccaaca*g*gcccacagatc-3′ and 5′-gctctgtgggcctgttggtgaagg-3′. The nucleotide substitutions, which are italicized, were introduced using the Quikchange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions and were confirmed by sequencing. The pCDNA3.1-MEKK3 mammalian expression construct was generated by reverse transcription-PCR using the primer pair 5′-gccgccatggacgaacaggaggcattg-3′ and 5′-gccgccatggacgaaacaggaggcattg-3′. The presence of full-length, wild-type MEKK3 sequence was confirmed by sequencing. The pDBleu-BRCA1 deletion constructs were generated by PCR of the relevant bases; *Not*I/*Sal*I restriction sites were included on the primers as described previously to facilitate cloning into pDBleu. The primer pairs for each construct were as follows: Del-1 (bases 4603–5373), 5′-agtccagtcagccaggtcatcccttcaaaatgcc-3′ and 5′-gagcgcgcggccgcgtctcgctttggaccttggtgg-3′; Del-2 (bases 5199–5711), 5′-agtccagtcagccgagtttgtgtgtgaacgg-3′ and 5′-gagcgcgcggccgcgtagtggctgtgggggatctgg-3′; Del-3 (bases 5010–5711), 5′-agtccagtcagccagtgtgagcagggagaagcc-3′ and 5′-gagcgcgcggccgcgtagtggctgtgggggatctgg-3′; and Del-4 (bases 4603–5543), 5′-agtccagtcagccaggtcatccccttcaaaatgcc-3′ and 5′-gagcgcgcggccgcgtcacaattgggtggacacc-3′.

Yeast Two-Hybrid Screen. The previously described Proquest system (Life Technologies, Rockville, MD) was used to perform the yeast two-hybrid screen (25). Briefly, the BRCA1 COOH-terminal motif, encoded by amino acids 1475–1863, was subcloned into the yeast DNA-binding domain vector, pDBleu, to generate pDBleu-BRCA1. Two additional constructs harboring point mutations within BRCA1 were generated by site-directed mutagenesis (Ala1708Glu and Met1775Glu; Refs. 1, 26). The competent yeast strain, MAV203, was cotransformed with 20 μg of the bait plasmid (pDBleu-BRCA1) and 20 μg of a fetal brain cDNA library (Life Technologies) cloned in the pPC86 vector. Self-activation of reporter genes by the BRCA1 construct alone was titrated out using 50 mM 3-amino-triazole. As a primary screen, interacting clones were selected based on their ability to activate the His3 reporter. His3-positive clones subsequently were tested to determine their ability to activate the LacZ and Ura3 reporters. Only clones that could activate all of the reporters were selected for retransformation assays as per the manufacturer's instructions.

Coimmunoprecipitation and Immunoblotting. Antibodies against BRCA1 included the COOH-terminal rabbit polyclonal antibodies C-20 (Santa Cruz Biotechnology, Santa Cruz, CA) and the N-terminal mouse monoclonal antibody AB-1 (Calbiochem, San Diego, CA). Antibodies against MEKK3 included a mouse monoclonal, clone-40 (BD Biosciences, San Jose, CA). V5-tagged MEKK3 expression constructs were detected using the mouse monoclonal antibody V5 (Invitrogen, Carlsbad, CA) against the synthetic V5 epitope. Isotype-matched control antibodies, including a rabbit control IgG (Santa Cruz Biotechnology) and a mouse control IgG1 (Calbiochem), were used where appropriate in the coimmunoprecipitation experiments. For coimmunoprecipitation experiments, total cellular lysate was extracted from MDA435 or HCC1937 cells in ELB low-salt buffer (250 mM NaCl, 0.1% Igepal, 50 mM HEPES, 5 mM EDTA, 0.5 mM DTT, and protease inhibitor mixture; Boehringer Mannheim, Indianapolis, IN). Lysates were passed through a 21-gauge needle and centrifuged, and protein concentration was determined by spectrophotometric readings at 594 nM using Bio-Rad protein assay reagent (Hercules, CA). Protein A-Sepharose beads were preassociated with the anti-MEKK3 monoclonal antibody, anti-BRCA1 (C-20), anti-V5 antibody, or the appropriate isotype-matched control antibody for 18 h at 4°C and then washed six times with ELB buffer. Total protein (2 mg) was incubated with the antibody complex for 4 h, followed by six washes with ELB buffer. Immunoblotting was performed using an anti-BRCA1 monoclonal antibody (AB-1) or the anti-MEKK3 monoclonal antibody. In the case of the kinase controls, immunoblotting was carried out with the anti-MEKK3 polyclonal antibody D-17.

Kinase Assays. Cellular lysates were extracted in PBS/0.1% Igepal + protease inhibitor mixture, lysed, and quantitated as described previously. Six hundred μg lysate were immunoprecipitated with the anti-MEKK3 monoclonal antibody for 3 h at 4°C. One-half of the immunoprecipitate was incubated with myelin basic protein (Sigma, St. Louis, MO) in the presence of γ^{32}P-ATP, and phosphorylated myelin basic protein was detected by autoradiography following electrophoresis on an 18% polyacrylamide gel. The remaining half of the immunoprecipitated protein lysate was used to determine equal sample loading by immunoblotting for MEKK3 protein using the anti-MEKK3 polyclonal antibody D-17. For kinase assays involving treatment with paclitaxel, MDA435, HCC-EV, or HCC-BR, cells were treated continuously for 4 or 24 h with 10 nM paclitaxel (Bristol-Myers Squibb, New York, NY), followed by extraction of protein lysate.

Phospho-Specific Western Blot Analysis. For phospho-specific p38/MAPK Western blot analyses, cells were uninduced or induced to express BRCA1 for 24 and 48 h, and protein lysates were collected in PBS + 10 mM sodium orthovanadate. Following centrifugation, the recovered cell pellet was snap frozen in liquid nitrogen and then resuspended in 50 μl modified RIPA buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% Igepal, 0.25% sodium deoxycholate, and 1 mM EGTA]. Following quantification, 20 μg protein were immunoblotted using a mouse monoclonal anti-phospho-p38 antibody (Invitrogen). Cells were treated with 20J/m^2 UV and lysates extracted after 24 h as a control for p38/MAPK activation.

SiRNA. MDA435 or MCF7 cells were transfected with a BRCA1-specific small interfering RNA (siRNA) oligonucleotide or a control oligonucleotide

for 24, 48, or 72 h followed by extraction of protein lysate. For siRNA experiments involving drug treatment, MCF7 cells were transfected with a BRCA1-specific siRNA oligonucleotide or a control oligonucleotide for 48 h, after which time cells were exposed to 10 nM paclitaxel for an additional 24 h; BRCA1 siRNA oligonucleotide, 5′-aac ctg tct cca caa agt gtg-3′; and control siRNA oligonucleotide, 5′-aaa acc cgu cua ggc ugu uac-3′.

RESULTS

Identification of MEKK3 as a BRCA1-Interacting Protein. To identify potential BRCA1-interacting proteins, we screened a fetal brain cDNA library (Life Technologies) with the COOH-terminal (amino acids 1475–1863) motif of BRCA1 using the Proquest yeast two-hybrid system (25). One hundred ten positives were identified that could activate the His reporter gene in a primary screen. Positive clones isolated based on activation of the His reporter gene then were replica plated onto agar plates containing X-gal (to assess for activation of LacZ), agar plates lacking Uracil (to assess activation of Ura3), and agar plates lacking Uracil in the presence of 5-fluorourotic acid. The product of the *Ura3* gene converts 5-fluorourotic acid to 5-fluorouracil, which is toxic to yeast cells providing negative selection in the presence of a positive interaction. Twenty-two clones were identified that could activate all of the three reporter genes and all of the four phenotypes, indicating that the majority of the original clones were likely to represent false positives. Two of these clones contained a 2.5-kb insert encoding the full-length open-reading frame of the serine/threonine kinase, MEKK3, an upstream regulator of the JNK/SAPK and p38/MAPK pathways (27). Fig. 1*A* illustrates four of the original 110 positive clones selected based on activation of the His reporter; only one (MEKK3) was capable of activating all of the four phenotypes. To determine whether BRCA1 disease-associated point mutations that have been shown previously to segregate with early-onset breast and ovarian cancer could abrogate the interaction with MEKK3 in yeast, we carried out retransformation assays. Constructs encoding either the breast cancer-associated point mutation Ala to Glu at amino acid 1708 within the first BRCT motif (26) or Arg to Met at amino acid 1775 within the second BRCT motif (1) were cotransformed into yeast with MEKK3 and assayed for activation of the individual reporter genes. Both BRCA1 disease-associated mutations abrogated the interaction with MEKK3 in yeast, suggesting that the BRCA1-MEKK3 interaction may be of physiologic relevance (Fig. 1*B*).

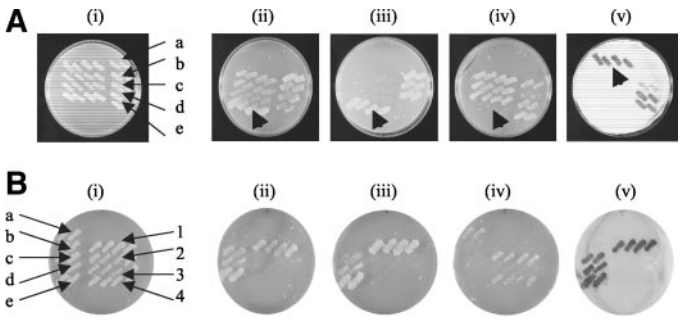

Fig. 1. BRCA1 and mitogen-activated protein kinase (MAPK) kinase kinase 3 (MEKK3) interact in yeast. *A*, yeast transformation assay illustrating four clones (each plated in quadruplicate) that were identified as being able to activate the His3 reporter in the presence of 50 mM 3-amino-triazole. The master plate (*i*) then was replica plated back onto selection plates to test for activation of (*ii*) the His3 reporter, (*iii*) the Ura3 reporter, (*iv*) the Ura3 reporter in the presence of 0.5% 5-fluorourotic acid (negative selection), and (*v*) the LacZ reporter. Only clone 4 (arrow) was capable of activation of the three reporter genes. Control strains (*a–e*) representing strong (*a*) to a no interaction control (*e*) are shown streaked from top to bottom on the right side of the plates (arrow). The clone containing MEKK3 is denoted by an arrow in each figure. *B*, retransformation assay evaluating the ability of disease-associated BRCA1 mutants to interact with MEKK3 in yeast. Yeast were transformed with pDBleu-BRCA1/pPC86-MEKK3 (*Row 1*), pDBleu-BRCA1 only (*Row 2*), pDBleu-BRCA1mut1 (ala5242glu)/pPC86-MEKK3 (*Row 3*), and pDBleu-BRCA1mut2 (met1775glu)/pPC86-MEKK3 (*Row 4*). Transformed colonies were streaked in quadruplicate on an SC-leu-trp master plate (*i*) and then replica plated onto selection plates to test for activation of the His3 reporter (*ii*), the Ura3 reporter (*iii*), the Ura3 reporter in the presence of 0.5% 5-fluorourotic acid (negative selection; *iv*), and the LacZ reporter (*v*). Only the full-length, wild-type BRCA1 construct, in combination with MEKK3-pPC86 (*Row 1*), was capable of activation of the three reporter genes. Control strains *a–e*, representing strong to a no interaction control, are shown streaked from bottom to top on the left side of each plate.

BRCA1 and MEKK3 Interact *in Vivo*. To determine whether MEKK3 interacted with BRCA1 *in vivo*, coimmunoprecipitation experiments were carried out following transfection of a V5-tagged MEKK3 construct into MDA435 cells, which express wild-type endogenous BRCA1. Anti-V5 immunoprecipitation of total cellular lysate from MDA435 cells coprecipitated endogenous BRCA1 (Fig. 2*A*, *Lane 1*). Anti-V5 immunoprecipitation of total cellular lysate from HCC1937 cells, which express a single copy of a COOH-terminal truncated BRCA1 protein product resulting in partial deletion of the second BRCT motif, failed to coprecipitate BRCA1 (Fig. 2*A*, *Lane 2*). Western blot analysis confirmed that V5-MEKK3 was expressed at equivalent levels in the MDA435 and HCC1937 cells following transfection (Fig. 2*A*), suggesting that the inability of V5-MEKK3 to coimmunoprecipitate BRCA1 was caused by mutant BRCA1 and not low transfection efficiency. Similarly, coimmunoprecipitation experiments in the MDA435 breast cancer cell line demonstrated that anti-BRCA1 immunoprecipitates contained endogenous MEKK3 (Fig. 2*B*, *Lane 1*). Consistent with that observed for V5-tagged MEKK3, endogenous MEKK3 failed to interact with BRCA1 in the HCC1937 cells (Fig. 2*C*, *Lane 3*). Additional experiments demonstrated reciprocal coprecipitation of endogenous BRCA1 from anti-MEKK3 immunoprecipitates derived from MDA435 cells (Fig. 2*D*). In agreement with the results described previously, endogenous BRCA1 failed to interact with MEKK3 in the BRCA1 mutant HCC1937 cells (Fig. 2*E*). Therefore, these data indicate that BRCA1 and MEKK3 interact *in vivo* and that the interaction is abrogated in the BRCA1 mutant HCC1937 cells.

Mapping of the MEKK3 Interaction Site on BRCA1. To define the minimal domain of BRCA1 required to mediate the BRCA1-MEKK3 interaction in yeast, we generated a series of BRCA1 COOH-terminal deletion constructs termed Del-1 (amino acids 1495–1746), Del-2 (amino acids 1694–1863), Del-3 (amino acids 1611–1863), and Del-4 (amino acids 1495–1803; Fig. 3, *A* and *B*). The individual deletion constructs were cotransformed into yeast with MEKK3 and assayed for activation of the individual reporter genes. Yeast also was retransformed with the full-length BRCT bait as a positive control. Apart from the full-length bait, only Del-3 (arrow) encoding amino acids 1611–1863, containing both BRCT motifs, was capable of interacting with MEKK3 and activating all of the three reporter genes (Fig. 3*C*). Furthermore, Del-4, which encodes the entire bait with the exception of the COOH-terminal amino acids 1803–1863 and missing part of the second BRCT motif (amino acids 1756–1855), failed to interact with MEKK3 in yeast, indicating that deleting as few as the last 60 amino acids abrogates the interaction (Fig. 3, *B* and *C*). This is consistent with the inability of MEKK3 to coimmunoprecipitate BRCA1 in the HCC1937 cells, which express a single copy of BRCA1 lacking the COOH-terminal, 33 amino acids. Furthermore, Del-2 encoding amino acids 1694–1863, lacking part of the first BRCT motif (amino acids 1642–1736), also fails to interact (Fig. 3, *B* and *C*), suggesting that the integrity of the BRCT motifs is critical to maintain the interaction with MEKK3. Therefore, the combined mapping data would indicate that amino acids 1611–1863, which encode BRCT motifs, are critical to maintain the BRCA1/MEKK3 interaction in yeast.

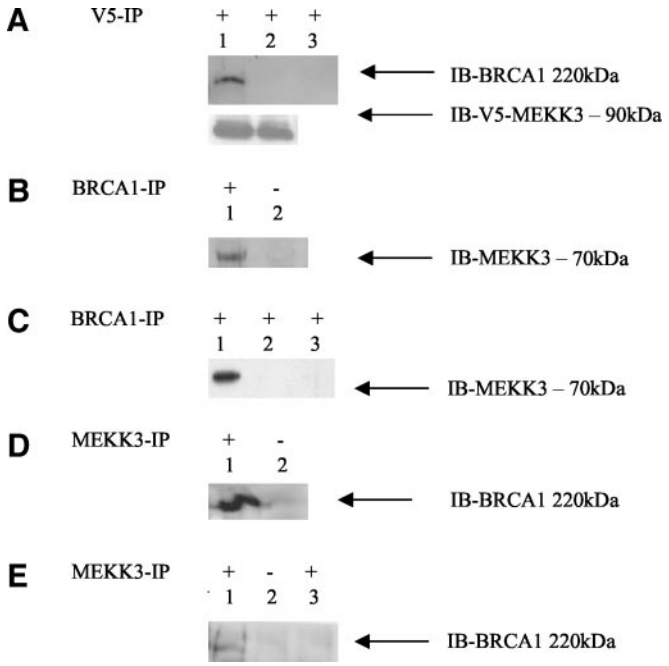

Fig. 2. BRCA1 and mitogen-activated protein kinase (MAPK) kinase kinase 3 (*MEKK3*) interact *in vivo*. *A*, V5-tagged MEKK3 coimmunoprecipitates BRCA1. Protein lysate (2 mg) was immunoprecipitated from MDA435 cells (*Lane 1*) or HCC1937 cells (*Lane 2*) following transfection with V5-MEKK3 or from MDA435 cells transfected with empty vector (*Lane 3*). Immunoprecipitations were carried out with a monoclonal antibody against V5 (*Lanes 1–3*). Immunoblotting (*IB*) was carried out using a monoclonal antibody, AB-1, to BRCA1. A 220-kDa band representing BRCA1 is present (*Lane 1*) but absent (*Lanes 2* and *3*), indicating that BRCA1 and MEKK3 interact in the MDA435 but not the HCC1937 cells. The vector-transfected control (*Lane 3*) is blank as expected. Equivalent expression levels of exogenous V5-MEKK3 were confirmed in the MDA435 and HCC1937 transfected cells by Western blot analysis using the V5 antibody. *B*, BRCA1 immunoprecipitates contain MEKK3. Protein lysate (2 mg) was immunoprecipitated from MDA435 cells (*Lanes 1* and *2*). Immunoprecipitations were carried out with the anti-BRCA1 polyclonal antibody C-20 (*Lane 1*) or with an isotype-matched control antibody (IgG; *Lane 2*). IB was carried out using a monoclonal antibody to MEKK3. A 70-kDa band representing MEKK3 is present (*Lane 1*) but not in the negative control (*Lane 2*). *C*, the BRCA1-MEKK3 interaction is abrogated in HCC1937 cells. Protein lysate (2 mg) was immunoprecipitated from MDA435 cells (*Lanes 1* and *2*) or from HCC1937 cells (*Lane 3*). Immunoprecipitations were carried out with the anti-BRCA1 monoclonal antibody AB-1 (*Lanes 1* and *3*) or with an isotype-matched control antibody (IgG1; *Lane 2*). IB was carried out using a monoclonal antibody to MEKK3. A 70-kDa band representing MEKK3 is present in *Lane 1* but absent in *Lanes 2* and *3*, indicating that BRCA1 and MEKK3 interact in the MDA435 cells but not in the BRCA1 mutant HCC1937 cells. *D*, MEKK3 immunoprecipitates contain BRCA1. Protein lysate (2 mg) was immunoprecipitated from MDA435 cells (*Lanes 1* and *2*). Immunoprecipitations were carried out with the anti-MEKK3 monoclonal antibody (*Lane 1*) or with an isotype-matched control antibody (IgG1; *Lane 2*). IB was carried out using a monoclonal antibody, AB-1, to BRCA1. A 220-kDa band representing BRCA1 is present in *Lane 1* but not *Lane 2*, indicating that MEKK3 immunoprecipitates contain BRCA1. *E*, the MEKK3-BRCA1 interaction is abrogated in HCC1937 cells. Protein lysate (2 mg) was immunoprecipitated from MDA435 cells (*Lanes 1* and *2*) or from HCC1937 cells (*Lane 3*). Immunoprecipitations were carried out with the anti-MEKK3 monoclonal antibody D-17 (*Lanes 1* and *3*) or with an isotype-matched control antibody (IgG1; *Lane 2*). IB was carried out using the BRCA1 monoclonal antibody, AB-1. A 220-kDa band representing BRCA1 is present in *Lane 1* but absent in *Lanes 2* and *3*, indicating that MEKK3 and BRCA1 interact in the MDA435 cells but not in the BRCA1 mutant HCC1937 cells.

MEKK3 Activity Is Modulated by BRCA1 Expression Levels. To investigate the biochemical significance of the BRCA1-MEKK3 interaction, we examined the effect of inducible BRCA1 expression on MEKK3 activity. MBR62-bcl2 cells, which are wild type for endogenous BRCA1, were induced to express exogenous BRCA1 by withdrawal of tetracycline for 24 h and examined by Western blot analysis to confirm BRCA1 induction (Fig. 4*A*). To determine the effect of inducible BRCA1 expression on MEKK3 activation, we carried out an *in vitro* kinase assay using myelin basic protein as a substrate. Inducible expression of BRCA1 resulted in significant activation of endogenous MEKK3 kinase activity (Fig. 4*B*). As a complementary approach, we inhibited endogenous BRCA1 expression using an siRNA approach in the MDA435 breast cancer cell line and examined the effect on MEKK3 kinase activity. Inhibition of endogenous BRCA1 expression was confirmed by immunoprecipitation and Western blot analysis (Fig. 4*C*) and resulted in a significant reduction of endogenous MEKK3 kinase activity (Fig. 4*D*). We had demonstrated previously that BRCA1 was a potent activator of the JNK kinase pathway but were unclear of the mechanistic basis of the observation (17). Because MEKK3 also has been reported to activate p38/MAPK via its downstream substrate MKK7, we analyzed the effect of BRCA1 induction on p38/MAPK activation (27). Similar to that observed for MEKK3, inducible expression of BRCA1 resulted in enhanced phosphorylation of endogenous p38/MAPK. Furthermore, the degree of p38/MAPK activation was comparable with that induced by UV irradiation, a potent known activator of p38/MAPK (Fig. 4*E*).

BRCA1 Is Required for Paclitaxel-Induced Activation of MEKK3. We have reported recently that BRCA1 dramatically sensitizes breast cancer cell lines to paclitaxel-induced apoptosis (21). Furthermore, it has been reported in the literature that paclitaxel is a potent activator of the JNK stress-response pathway (28, 29). Therefore, we postulated that BRCA1 might play an important role in mediating activation of MEKK3 in response to this agent. To investigate this further, we first demonstrated that paclitaxel could activate MEKK3 activity because this had not been reported previously. *In vitro* kinase assays demonstrated that treatment of MDA435 cells with 10 nM paclitaxel resulted in potent activation of MEKK3 activity 24 h following treatment (Fig. 5*A*, *Lane 4*). Paclitaxel-induced activation of MEKK3 activity was comparable with that induced 24 h following exposure of MDA435 cells to 20 J/m^2 UV irradiation, one of the most potent known activators of this pathway (Fig. 5*A*, *Lane 2*). We used two complementary approaches to evaluate the role played by BRCA1 in modulating the activity of MEKK3. In the first instance, we used an siRNA approach to inhibit endogenous BRCA1 expression and evaluated the ability of paclitaxel to activate endogenous MEKK3 activity.

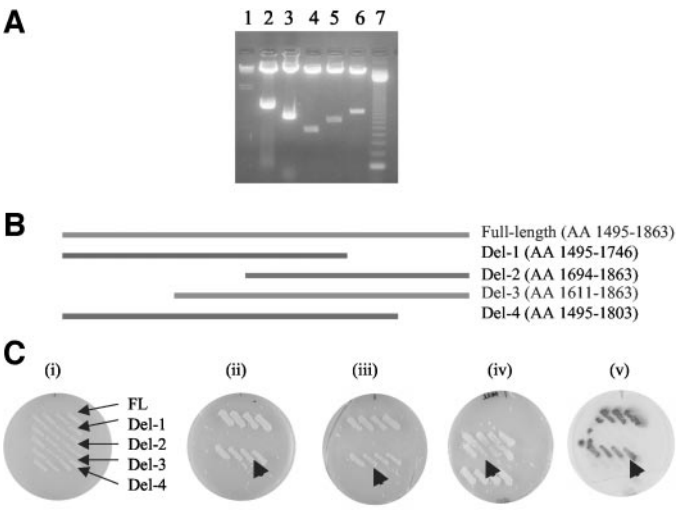

Fig. 3. Mapping of the mitogen-activated protein kinase (MAPK) kinase kinase 3 (MEKK3) interaction motif on BRCA1. *A*, a series of BRCA1 deletion constructs were generated by PCR. Full-length bait (*FL*) encoding amino acids 1495–1863 (*Lane 2*). Del-1, encoding amino acids 1495–1746 (*Lane 3*). Del-2, encoding amino acids 1694–1863 (*Lane 4*). Del-3, encoding amino acids 1611–1863 (*Lane 5*), and Del-4, encoding amino acids 1495–1803 (*Lane 6*). DNA markers are included (*Lanes 1* and *7*). *B*, a schematic outlining the FL bait construct and the respective deletion constructs. *C*, yeast retransformation assay in which FL bait and the four deletion constructs (*Del-1–4*) were cotransformed with pPC86-MEKK3 into yeast and evaluated for activation of the reporter genes. Transformed colonies were streaked in quadruplicate on an SC-leu-trp master plate (*i*) and then replica plated onto selection plates to test for activation of the His3 reporter (*ii*), the Ura3 reporter (*iii*), the Ura3 reporter in the presence of 0.5% 5-fluorourotic acid (negative selection; *iv*), and the LacZ reporter (*v*). Only FL bait and Del-3 (arrow) were capable of activation of the three reporter genes, indicative of an interaction with MEKK3.

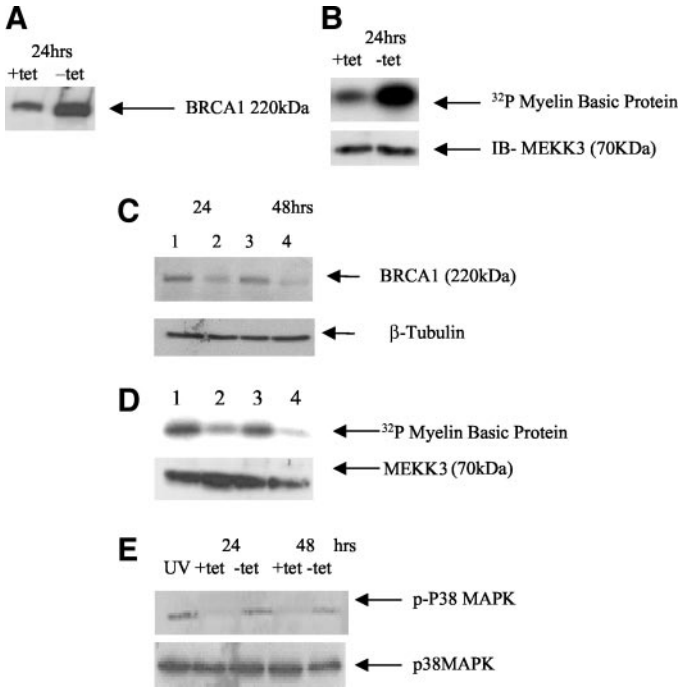

Fig. 4. Mitogen-activated protein kinase (*MAPK*) kinase kinase 3 (*MEKK3*) activity is regulated by BRCA1 expression levels. *A*, Western blot analysis demonstrating inducible expression of BRCA1 24 h following withdrawal of tetracycline in the BRCA1 wild-type MBR62-bcl2 cells (+*tet*, BRCA1 off; −*tet*, BRCA1 on). *B, top, in vitro* kinase assay. Endogenous MEKK3 was immunoprecipitated from MBR62-bcl2 cells grown in the presence of tetracycline (inducible BRCA1 off) or absence of tetracycline (BRCA1 induced) for 24 h and used to phosphorylate myelin basic protein. MEKK3 activity is elevated dramatically 24 h following inducible expression of BRCA1 (−tet). *Bottom*, Western blot analysis is shown to demonstrate comparable amounts of immunoprecipitated kinase (MEKK3). *C*, immunoprecipitation and Western blot analysis demonstrating a marked reduction in exogenous BRCA1 protein levels in MDA435 cells transfected with a BRCA1-specific small interfering RNA (siRNA) oligonucleotide (*Lanes 2* and *4*) compared with a control siRNA oligonucleotide (*Lanes 1* and *3*). A β-tubulin Western blot analysis was carried out using the identical protein lysate as a control for the specificity of the BRCA1 siRNA. *D, top, in vitro* kinase assay demonstrating that inhibition of endogenous BRCA1 results in reduced MEKK3 activity. Endogenous MEKK3 was immunoprecipitated from MDA435 cells transfected with a scrambled control oligonucleotide (*Lanes 1* and *3*) or a BRCA1-specific siRNA oligonucleotide (*Lanes 2* and *4*) for 24 h (*Lanes 1* and *2*) and 48 h (*Lanes 3* and *4*) and used to phosphorylate myelin basic protein. MEKK3 activity is dramatically reduced in MDA435 cells transfected with the BRCA1-specific siRNA oligonucleotide at 24 h (*Lane 2*) and 48 h (*Lane 4*). *Bottom*, Western blot analysis is shown to demonstrate comparable amounts of immunoprecipitated kinase (MEKK3). *E, top*, anti-phospho-p38/MAPK Western blot analysis demonstrating maximal activation of p38/MAPK 24 h following inducible expression of BRCA1 (−tet) in the MBR62-bcl2 cells. Uninduced MBR62-bcl2 cells were treated with 20 J/m^2 UV irradiation 4 h before protein extraction as a positive control for p38/MAPK activation. *Bottom*, Western blot analysis demonstrating equal amounts of p38/MAPK protein in the presence or absence of inducible BRCA1 expression.

MDA435 cells were transfected with a scrambled oligonucleotide or a BRCA1-specific siRNA oligonucleotide for 48 h and then treated with 10 nM paclitaxel for 24 h. Consistent with Fig. 5*A*, we demonstrated activation of MEKK3 activity in cells transfected with the control oligonucleotide 24 h following paclitaxel treatment (Fig. 5*B*, *Lane 3*). In contrast, transfection of cells with the BRCA1-specific siRNA oligonucleotide completely abrogated paclitaxel-induced activation of MEKK3 (Fig. 5*B, Lane 4*). To confirm inhibition of endogenous BRCA1 expression levels, we carried out immunoprecipitation and Western blot analysis from identical lysates, demonstrating an 80% reduction in levels in cells transfected with the BRCA1-specific siRNA oligonucleotide compared with scrambled control (Fig. 5*C*). As an alternative approach, we evaluated the ability of paclitaxel to activate MEKK3 in the BRCA1 mutant HCC1937 cells stably transfected with empty Rc/CMV vector (HCC-EV) or reconstituted with full-length exogenous wild-type BRCA1 in Rc/CMV (HCC-BR). *In vitro* kinase assays demonstrated higher background levels of MEKK3 activity in the BRCA1-reconstituted cells (Fig. 5*D, Lane 2*). Furthermore, treatment of HCC-EV cells with 10 nM paclitaxel for 24 h failed to activate MEKK3 but resulted in substantially enhanced activation of MEKK3 in HCC-BR cells (Fig. 5*D*, compare *Lanes 3* and *4*). Therefore, these combined data suggest that BRCA1 is required for paclitaxel-induced activation of MEKK3. To determine whether inactivation of BRCA1 modulates paclitaxel-induced apoptosis, we carried out dose-response and caspase-3 cleavage assays in the presence and absence of paclitaxel in the HCC-EV and HCC-BR cells. The HCC-EV and HCC-BR cells were plated at equal densities and exposed to a range of concentrations of paclitaxel for 72 h, after which time cell counts were carried out. Reconstitution of BRCA1 in the HCC1937 cells resulted in a 1000-fold increase in sensitivity of the HCC-BR cells to paclitaxel, with the IC_{50} value decreasing from 6.21 μM in the HCC-EV cells compared with 7.73 nM in the HCC-BR cells (Fig. 5*E*). Consistent with these findings, we demonstrated that treatment of HCC-BR cells with paclitaxel resulted in significantly enhanced expression of cleaved caspase-3 compared with HCC-EV cells (Fig. 5*F*), indicating that BRCA1 sensitized HCC-BR cells to paclitaxel-induced apoptosis.

DISCUSSION

In the current study we have demonstrated a specific interaction between BRCA1 and MEKK3, an upstream regulator of the JNK/SAPK and p38/MAPK pathways. This interaction was abrogated in yeast by BRCA1 disease-associated mutations (Fig. 1*B*) and in the BRCA1-deficient HCC1937 cells (Fig. 2*B*), suggesting that it may be physiologically relevant. Inducible expression of BRCA1 activated MEKK3 (Fig. 4, *A* and *B*), and conversely, inhibition of endogenous BRCA1 using an siRNA-based approach inhibited MEKK3 kinase activity (Fig. 4, *C* and *D*). Furthermore, we demonstrated that paclitaxel is a potent inducer of MEKK3 activity and that this effect is dependent on functional BRCA1 (Fig. 5). Together, these data suggest that BRCA1 may represent an important mediator of the MEKK3/JNK/p38/MAPK paclitaxel stress-response pathway (Fig. 6).

The observation that MEKK3 fails to interact with BRCA1 in HCC1937 cells suggests that this interaction may be physiologically relevant and provides a rationale to explain the failure of paclitaxel to induce activation of MEKK3 in this cell line. The HCC1937 cells encode a single copy of BRCA1 that is missing the COOH-terminal 33 amino acids, resulting in disruption of the second BRCT motif (12). This would suggest that the integrity of the extreme COOH-terminus including the second BRCT motif is essential to maintain the interaction with BRCA1. Mapping of the BRCA1-MEKK3 interaction site in yeast (Fig. 3) supports the importance of the COOH-terminus for maintaining the interaction. Significantly, a deletion construct lacking the COOH-terminal 60 amino acids (Del-4) also failed to reconstitute the interaction in yeast, confirming our observations in the HCC1937 cells. We also have demonstrated that in yeast the BRCA1 disease-associated point mutations, C to A at nucleotide 5243 (26) and T to G at nucleotide 5443 (1), also abrogate the interaction with MEKK3. Interestingly, both these mutations fall within the BRCT motifs, suggesting that disruption of either domain abrogates the interaction with MEKK3 and highlighting the potential importance of these motifs in regulating the paclitaxel stress-response pathway. BRCT motifs have been identified recently as phosphopeptide-binding domains, and a potential consensus sequence has been identified that preferentially binds to the BRCA1 BRCT motifs (30–32). The optimal phospho-binding motif included a pSer or pThr with an extremely strong selection in the +3 position for aromatic and aliphatic residues. Interestingly, MEKK3 contains a closely conserved motif exhibiting a phospho-threonine residue at amino acid 74 with

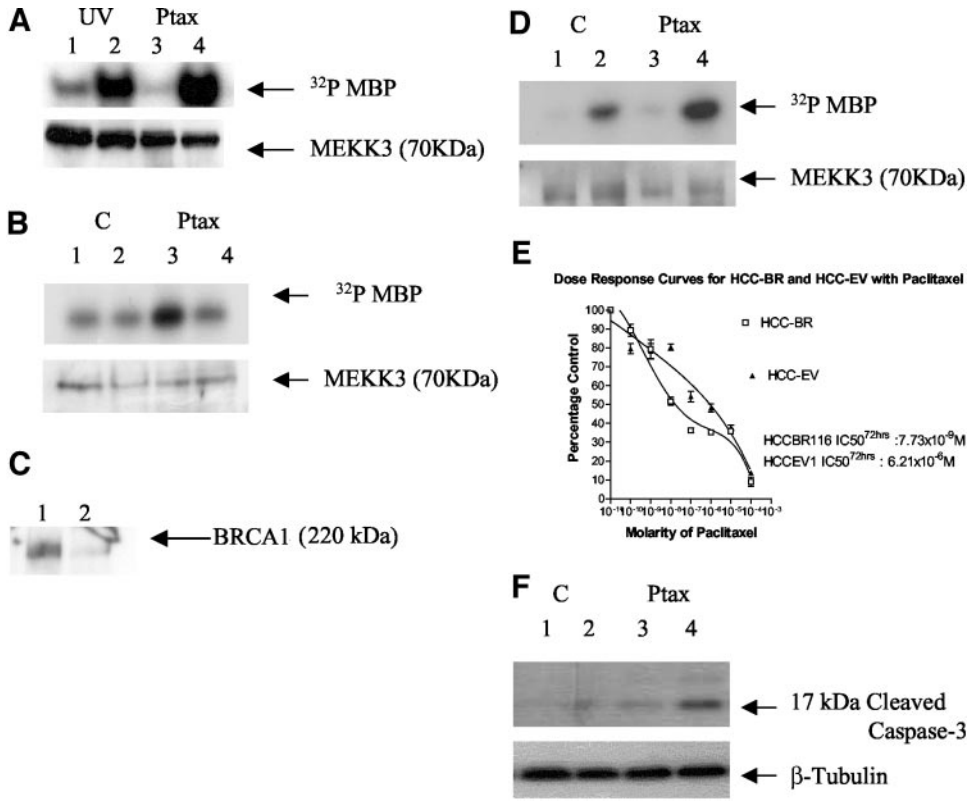

Fig. 5. BRCA1 is required for paclitaxel-induced activation of mitogen-activated protein kinase (MAPK) kinase kinase 3 (*MEKK3*). *A, top, in vitro* kinase assay demonstrating comparable activation of MEKK3 in the BRCA1 wild-type MDA435 cells following treatment with UV and paclitaxel for 24 h. Endogenous MEKK3 was immunoprecipitated from untreated MDA435 cells (*Lanes 1* and *3*) or following treatment with 20 J/m^2 UV (*Lane 2*) or 10 nM paclitaxel (*Lane 4*) for 24 h and used to phosphorylate myelin basic protein. *Bottom,* MEKK3 Western blot analysis demonstrating equal amounts of MEKK3 protein in each lane. *B, in vitro* kinase assay comparing the ability of paclitaxel to induce activation of MEKK3 in MDA435 cells transfected with a BRCA1-specific small interfering RNA (siRNA) oligo (*Lanes 2* and *4*) compared with a scrambled control oligo (*Lanes 1* and *3*). Endogenous MEKK3 was immunoprecipitated from MDA435 cells that had been untreated (*Lanes 1* and *2*) or treated with 10 nM paclitaxel (*Lanes 3* and *4*) for 24 h and used to phosphorylate myelin basic protein. *Bottom,* MEKK3 Western blot demonstrating comparable amounts of MEKK3 protein in each lane. *C,* immunoprecipitation and Western blot analysis demonstrating inhibition of endogenous BRCA1 protein 72 h following transfection of MDA435 cells with a BRCA1-specific siRNA oligo (*Lane 2*) compared with a scrambled control oligo (*Lane 1*). *D, top, in vitro* kinase assay demonstrating enhanced activation of MEKK3 in HCC-BR cells compared with HCC-EV cells following paclitaxel treatment. Endogenous MEKK3 was immunoprecipitated from untreated HCC-EV (*Lane 1*) and HCC-BR (*Lane 2*) cells or following treatment of HCC-EV cells (*Lane 3*) and HCC-BR cells (*Lane 4*) for 24 h with 10 nM paclitaxel. The immunoprecipitated lysate was used to phosphorylate myelin basic protein in an *in vitro* kinase assay. *Bottom,* Western blot analysis is shown to demonstrate comparable amounts of immunoprecipitated kinase (MEKK3). *E,* dose-response curve demonstrating a >1000-fold increase in sensitivity of HCC-BR cells to paclitaxel compared with HCC-EV following treatment with paclitaxel (▲, HCC-EV; □, HVV-BR). *F, top,* caspase-3 cleavage assay demonstrating enhanced apoptosis in HCC-BR compared with HCC-EV cells following treatment with 10 nM paclitaxel. Total cellular lysate was extracted from HCC-EV (*Lanes 1* and *3*) and HCC-BR cells (*Lanes 2* and *4*) that had been untreated (*Lanes 1* and *2*) or treated with 10 nM paclitaxel (*Lanes 3* and *4*). *Bottom,* anti-β-tubulin Western blot analysis is shown to demonstrate that comparable amounts of protein lysate were loaded in each well.

conserved valine and phenylalanine residues at the +3 and +4 positions, respectively. Furthermore, the authors of these studies demonstrated that both BRCA1 BRCT motifs were essential to mediate an interaction with the consensus phosphopeptide and suggest that the two BRCT motifs function as a single interaction module. This observation is consistent with our own mapping studies, which demonstrated that both BRCT motifs were required to sustain an interaction with MEKK3.

This study also supports our previous observations that BRCA1 is a potent activator of JNK/SAPK and provides a potential pathway through which this occurs (17). However, the exact mechanism through which BRCA1 regulates the activation of the MEKK3 pathway is a matter for speculation. It has been reported previously that BRCA1 is required for activation of the CHK1 kinase following ionizing radiation through an unknown mechanism, resulting in activation of the G_2-M checkpoint (33). Similarly, it has been reported that BRCA1 facilitates the ability of ATM and ATR to phosphorylate specific downstream target genes associated with cell cycle arrest and apoptosis (24). In the current study, we demonstrate that BRCA1 is required for paclitaxel-induced activation of MEKK3, suggesting that BRCA1 may mediate activation of different kinase pathways following specific types of cellular stress. Because BRCA1 itself does not possess any intrinsic kinase activity, it is likely that BRCA1 facilitates paclitaxel-induced activation of MEKK3 through alternative mechanisms. It is possible that BRCA1 acts as a scaffold protein, bringing various components of the stress-response cascade into close contact thereby facilitating their activation. This type of mechanism already has been well described for the JNK/SAPK pathway, where the JIP protein family has been shown to bind to JNK and MKK7 and potentiate JNK activation by mixed-lineage protein kinases (34). Interestingly, like BRCA1, the JIP proteins have been implicated in transcriptional regulation, and JIP1 has been shown to bind to and activate the GLUT2 promoter (35). MP1 also has been identified as a scaffold protein that can bind to MEK1 and extracellular signal-regulated kinase 1 and facilitate their activation (36). It has been suggested recently that different scaffold proteins function to add specificity to MAPK signaling pathways, allowing typically promiscuous kinases to target specific downstream substrates (37). Therefore, it is intriguing to speculate that BRCA1 also may act as a molecular scaffold that functions to regulate apoptotic or cell cycle arrest programs in response to cellular stress, such as damage to the mitotic spindle. We have reported recently that BRCA1 modulates G_2-M arrest and apoptosis in response to paclitaxel and vinorelbine (21). Furthermore, JNK and p38/MAPK lie downstream of MEKK3

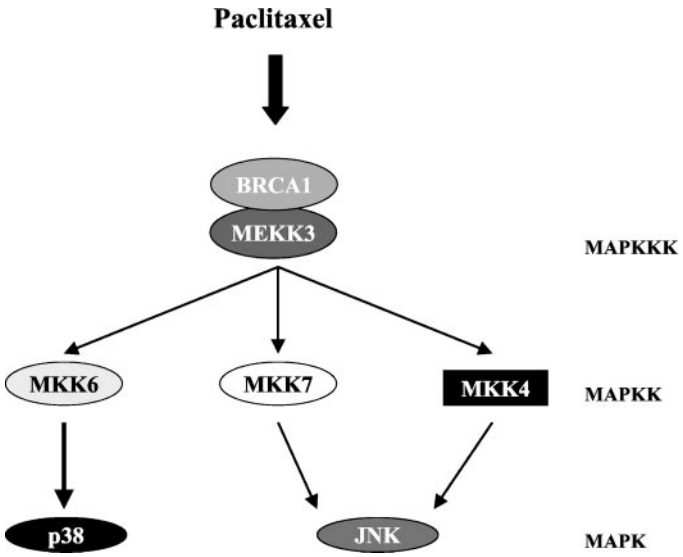

Fig. 6. Schematic outlining a potential role for BRCA1 in mediating paclitaxel-induced activation of c-Jun N-terminal kinase (*JNK*) and p38/mitogen-activated protein kinase (*MAPK*).

(Fig. 6), and both have been implicated in mediating apoptosis in response to paclitaxel treatment (28, 29, 38, 39). We have reported previously that BRCA1 sensitizes breast cancer cells to paclitaxel-induced apoptosis (21). Therefore, the observation that BRCA1 binds MEKK3 and is required for its activation following treatment of cells with paclitaxel provides a potential mechanism through which BRCA1 regulates the cellular response to antimicrotubule agents. The current study raises additional speculation as to how patients with BRCA1-associated tumors should be treated and suggests that theses tumors may not respond to agents, such as paclitaxel, that disrupt the mitotic spindle.

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BRCA1 Interacts with and Is Required for Paclitaxel-Induced Activation of Mitogen-Activated Protein Kinase Kinase Kinase 3

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