A Novel Tricomplex of BRCA1, Nmi, and c-Myc Inhibits c-Myc-induced Human Telomerase Reverse Transcriptase Gene (hTERT) Promoter Activity in Breast Cancer*

Huchun Li, Tae-Hee Lee, and Hava Avraham‡

From the Division of Experimental Medicine, Beth Israel Deaconess Medical Center, Harvard Institutes of Medicine, Boston, Massachusetts 02115

Germ-line mutations in BRCA1 predispose individuals to breast and ovarian cancers. We observed a novel endogenous association of BRCA1 with Nmi (N-Myc-interacting protein) in breast cancer cells. Nmi was found to interact specifically with BRCA1, both in vitro and in vivo, by binding to two major domains in BRCA1, amino acid residues 298–693 and 1301–1893. Homodimerization of Nmi enhanced its association with BRCA1. Nmi functioned as an adaptor molecule to recruit c-Myc to a complex containing Nmi-c-Myc-BRCA1. Because c-Myc can activate transcription of the human telomerase reverse transcriptase gene (hTERT), we addressed the role of BRCA1 and Nmi in modulating c-Myc-induced hTERT promoter activity. Although Nmi or BRCA1 alone had no effect on c-Myc induced hTERT promoter activity, BRCA1 together with Nmi significantly inhibited this c-Myc induced hTERT promoter activity (~75% inhibition). Two mutated forms of BRCA1, a missense (A1708E) and a nonsense (Y1853X) that have been identified in familial breast cancers, associated with Nmi and c-Myc but failed to suppress c-Myc-induced hTERT promoter activity. These results demonstrate a novel pathogenic mechanism whereby mutations in BRCA1, via a novel transcription factor complex containing BRCA1, c-Myc, and Nmi, impair inhibition of c-Myc-induced hTERT promoter activity, which allows sustained activation of telomerase, a key enzyme in carcinogenesis.

Germ-line mutations in the tumor suppressor BRCA1 predispose women to breast and ovarian cancers. Current evidence demonstrates that mutations in BRCA1 do not directly result in tumor formation but instead cause genetic instability, subjecting cells to a high risk of malignant transformation (1–3). The emerging roles of BRCA1 in regulating transcription, repair of DNA damage, and the cell cycle checkpoint indicate that its functions are broad and complex. The BRCA1 gene encodes an 1863-amino acid (220 Kγ) protein that is targeted to the nucleus by nuclear localization sequences (amino acids 503–508 and 606–615), which interact with the nuclear transport signal receptor (4–7). BRCA1 has an N-terminal ring finger domain and a C-terminal transcriptional activation domain that activates transcription when linked to a Gal4 DNA-binding domain (8, 9). In addition, BRCA1 can function as a transcriptional repressor (10), and overexpression of BRCA1 inhibits the growth of breast and ovarian cancer cells (11). BRCA1 has intrinsic transactivation activity and is able to activate the p21 promoter (12). Moreover, BRCA1 is linked to a number of genes involved in transcriptional regulation, including p53, CtIP, c-myc, the RNA polymerase II holoenzyme complex, and histone deacetylase complex (13–19). BRCA1 was also shown to repress estradiol-responsive ER-α-mediated transcriptional activity (20).

c-myc is a proto-oncogene that is implicated in tumorigenesis, embryonic development, and apoptosis (16, 21–27). BRCA1 was found to interact with c-Myc and affect cellular phenotypes caused by the synergistic actions of c-Myc and Ras (16). BRCA1 inhibits the transcriptional activity of c-Myc at the CDC25A promoter and reverses the cellular transformation activity of c-Myc. Efficient BRCA1-c-Myc association requires the intact helix-loop-helix region of c-Myc, a motif involved in c-Myc-Max dimerization. However, BRCA1 does not bind to Max (16). Interestingly, c-Myc activates the hTERT gene promoter leading to telomerase activation (22, 27). Telomerase maintenance has been proposed as an essential prerequisite to human tumor development (28–30). However, the molecular mechanisms that activate this enzyme during neoplastic transformation are not well understood.

To identify potential novel binding partner(s) of BRCA1, which may help to elucidate its role(s) in hereditary breast cancer, we performed yeast two-hybrid screening using the C-terminal domain (1301–1853 aa)† of BRCA1 as a bait. Here, we report that Nmi (N-Myc and c-Myc interacting protein) interacts in vivo and in vitro with BRCA1 and c-Myc and that this complex down-regulates c-Myc-induced hTERT transcription in breast cancer cells.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—LexA-BRCA1 (1301–1853 aa) was constructed by PCR. A Jurkat LexA cDNA library (CLONTECH) was screened using LexA-BRCA1 (1301–1853 aa) as a bait. Among the 1 × 10⁶ CDNA libraries screened, 15 clones were identified as LexA-BRCA1 (1301–1853 aa)-interacting partners in both the β-galactosidase re

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* This work was supported in part by National Institutes of Health Grants HL61456, CA76226, and R21CA87290, Department of the Army Grants DAMD17-98-1-8032 and DAMD17-99-1-9078, Experienced Breast Cancer Research Grant 34080057089, a Massachusetts Department of Public Health grant, the Milheim Foundation, and a Claudia Sargent Memorial Breast Cancer Fellowship (to H. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ This work was done during the term of an established investigatorship from the American Heart Association. To whom correspondence should be addressed: Division of Experimental Medicine, Beth Israel Deaconess Medical Center, Harvard Institutes of Medicine, 4 Blackfan Circle, Boston, MA 02115. Tel.: 617-667-0073; Fax: 617-975-5240 or 617-975-6373, E-mail: havrah@caregroup.harvard.edu.

† The abbreviations used are: aa, amino acid(s); hTERT, human telomerase reverse transcriptase gene; Nmi, N-Myc-interacting protein; 4-OHT, 4-hydroxytamoxifen; IFN-γ, interferon γ; STAT, signal transducers and activators of transcription; HA, hemagglutinin; GST, glutathione S-transferase; ER, estrogen receptor; BRCT, BRCA1 C terminus repeat; Max, Myc-associating protein X.
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**Fig. 1. BRCA1-Nmi interaction in vitro and in vivo.** A and B, 293T cells were transfected with Flag-Nmi and HA-BRCA1 as indicated. Cells were transfected with 0.5 μg of HA-BRCA1 DNA plasmid and 0.375 μg of Flag-Nmi DNA plasmid. After 48 h, total cell lysates were collected and immunoprecipitated (IP) with anti-HA antibody (A) or anti-Flag antibody (B). The immunocomplexes were then separated by SDS-PAGE and detected by Western blotting (WB) using anti-Flag or anti-HA antibodies as indicated. Aliquots of the lysates were analyzed for expression level of Flag-Nmi and HA-BRCA1 by Western blotting. Input, total transfected cell lysates. C and D, to demonstrate the endogenous association of BRCA1 and Nmi, total cell lysates from T47D cells were immunoprecipitated with BRCA1 antibody (C), Nmi antibody (D), or control antibody. The precipitated proteins were separated by SDS-PAGE and then detected by Western blotting (WB) using either Nmi (C) or BRCA1 (D) antibodies. In the lower panels, the blots were stripped and reprobed with anti-BRCA1 and anti-Nmi antibodies, respectively. E, Nmi and BRCA1 co-localized in nuclear dots in T47D cells, MCF-7 cells, and MDA-MB-231 cells. Cells were dually stained with BRCA1 monoclonal antibody (green) and affinity-purified rabbit polyclonal antiserum to Nmi (red) as described under “Experimental Procedures.” Significant co-localization of BRCA1 and Nmi nuclear dots in cells is reflected by the presence of yellow nuclear dots in the merged images.

porter and leucine reporter assays. One of the candidates coded for the N terminus truncated Nmi (amino acids 91–307).

**Cells and Reagents—**293T cells, MDA-MB-231 cells, and NIH 3T3/c-Myc-ER (24) cells were maintained in Dulbecco’s modified Eagle’s medium, 10% fetal bovine serum, penicillin/streptomycin (100 U/ml), and 0.29 g/ml L-glutamine. T47D and MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI medium, 10% fetal bovine serum, 10 μg/ml insulin, penicillin/streptomycin (100 U/ml), and L-glutamine (0.29 μg/ml). MCF-10A cells were obtained from the American Type Culture Collection. The cells were maintained in a 1:1 mixture of Ham’s F12 medium and Dulbecco’s modified Eagle’s medium containing 5% horse serum, 2.5 mM l-glutamine, 20 ng/ml epidermal growth factor, 100 ng/ml cholaer toxin, 0.01 ng/ml insulin, and 500 ng/ml hydrocortisone. The HA-BRCA1 cDNA construct was kindly provided by Dr. Scully (31). HA-Nmi was a gift from Dr. Zervas (32). pEBC-c-Myc and pRc-c-Myc were described previously (32).

The human telomerase catalytic subunit promoter (hTERT) (core 181 bp region) was a gift from Dr. Kyo (33). HA-BRCA1 (1–893 aa) and HA-BRCA1 (1301–1863 aa) were constructed by inserting individual PCR fragments into a pcDNA3 vector (30). HA-BRCA1 mutants (A1708E, Y1853X) were generated by using the QuickChange™ site-directed mutagenesis kit (Stratagene). All new constructs were confirmed by sequencing. The Flag-Nmi cDNA construct was generated by PCR. GST-BRCA1 fusion cDNA constructs were generated by cloning individual BRCA1 PCR products into a pGEX4T-1 plasmid vector. Monoclonal anti-HA antibody (HA.11), anti-Flag antibody (M2), and anti-c-Myc antibodies (N-262 and 9E10) were purchased from Babco, Sigma, and Santa Cruz Biotechnology, respectively. BRCA1 monoclonal antibodies (BR1N129.5 and BR1H945.2) were a gift from Dr. Tina Kuus Reichel (Beckman-Coulter). Nmi antibodies (R1373 and R1445) were obtained from Dr. W. J. Leonard (34).

Immunoprecipitation—293T cells were transfected with the indicated plasmids using LipofectAMINE Plus reagent (Invitrogen). After a 48-h transfection, cells were lysed in 600 μl of lysis buffer (50 mM Tris/HCl, pH 7.6, 1% Triton X-100, 150 mM NaCl, 0.1 mM EDTA, 10% glycerol, and protease inhibitor mixture) and put on ice for 15 min. After centrifugation for 15 min at 4°C, cell lysates were preincubated with protein G-Sepharose beads for 1 h at 4°C. Immunoprecipitations were performed at 4°C for 2 h with the indicated antibodies, and beads were washed four times with lysis buffer. Immunoprecipitates were subjected to SDS-PAGE followed by autoradiography. Association assays between the GST-c-Myc fusion protein and BRCA1 or Nmi were performed by incubating GST-beads with 293T total cell lysates for 2 h at 4°C.

Immunostaining—Cells were grown on coverslips, fixed with 3% paraformaldehyde/2% sucrose in phosphate-buffered saline for 10 min, and permeabilized with 0.5% Triton X-100. Cells were then incubated with mouse monoclonal antibodies to BRCA1 (at 1:200) and polyclonal anti-α-case to Nmi (at 1:500) in phosphate-buffered saline with 10% goat serum. After washing, appropriate species-specific, fluorochrome-conjugated secondary antibodies (Jackson Immunoresearch Laboratories) were applied as recommended by the manufacturer, and fluorescence was visualized using a confocal microscope. For staining of c-Myc and Nmi, MDA-MB-231 cells were transfected with pRc-c-Myc plasmid followed by immunostaining with anti-c-Myc monoclonal antibody (9E10) and anti-Nmi rabbit polyclonal antibody (R1445).

GST Pull-down Assay—Expression and purification of GST fusion proteins were performed following the manufacturer’s manual (Amer sham Biosciences). The GST pull-down assay was performed by incubating 10 μg of GST fusion protein bound to glutathione-Sepharose beads with the total cell lysates of 293T cells transfected with the indicated expression plasmids in 600 μl of lysis buffer (50 mM Tris/HCl, pH 7.6, 1% Triton X-100, 150 mM NaCl, 0.1 mM EDTA, 10% glycerol, and protease inhibitor mixture) for 1 h at 4°C. After four washes of the beads in lysis buffer, the precipitates were subjected to SDS-PAGE followed by autoradiography.

 Luciferase Assays—Transfections in T47D cells were performed by using LipofectAMINE Plus reagent (Invitrogen) with 0.5 μg of the hTERT promoter (181 bp) plasmid, 0.5 μg of the pmCMVβ-gal plasmid, 0.375 μg of the pRc-c-Myc plasmid, 0.75 μg of the wild-type or mutant HA-BRCA1 plasmids, and 0.5 μg of the Flag-Nmi plasmid following adjustment of total DNA amounts with a pcDNA3 vector in 6-well plates. Cells were harvested 48 h after transfection, and luciferase assays were performed. Transfection efficiency was assessed by determining β-galactosidase activity, and luciferase activities were normalized by β-galactosidase activities. NIH 3T3/c-Myc-ER cells were transfected with 0.5 μg of the hTERT promoter (181 bp), 0.5 μg of HA-BRCA1, 0.25 μg of Flag-Nmi, and 0.5 μg of pmCMVβ-gal plasmids using
LipofectAMINE Plus reagent (Invitrogen). After a 6-h transfection, cells were treated with 0.75 μM 4-OHT for 48 h, and luciferase and β-galactosidase assays were performed.

RESULTS

BRCA1 Interacts with Nmi in Vitro and in Vivo—The BRCT domain (1301–1853 aa) of BRCA1 served as bait in the LexA yeast two-hybrid system (CLONTECH). One gene identified in this analysis encoded amino acids (91–307 aa) of the known protein Nmi. Nmi was identified as an N-Myc/c-Myc-interacting protein (32). Although the function of Nmi is unknown, its C terminus is similar to an interferon-induced leucine zipper protein, IFP 35 (35). Nmi binds to both c-Myc and N-Myc in cells and to other transcription factors in yeast. It is expressed at low levels in all fetal and adult human tissues (32) except the brain and is seen at high levels in certain myeloid leukemias, which also express high levels of c-Myc (32). Nmi protein can be induced by interleukin-2 or IFN-γ in lymphocytes (36, 37). Nmi was also shown to interact with all STAT proteins except Stat2 to enhance the association of the CBP/p300 coactivator protein with Stat1 and Stat5, and together with CBP (CREB-binding protein/p300, to augment interleukin-2 and IFN-γ-dependent transcription (34). To test whether Nmi directly binds to the BRCT repeat, in vitro binding assays were performed using a GST-BRCA1 (1301–1863 aa) fusion protein. Bacterially expressed and purified GST-BRCA1 (1301–1863 aa), but not GST alone, bound to the in vitro synthesized [35S]methionine-labeled Nmi protein (data not shown). To confirm the BRCA1-Nmi interaction in mammalian cells, we co-transfected human 293T embryonic kidney cells with hemagglutinin (HA) epitope-tagged BRCA1 and Flag-tagged Nmi. Forty-eight hours after transfection, cell lysates were prepared and subjected to immunoprecipitation with anti-HA antibody. The precipitated complex was then resolved on SDS-PAGE followed by Western blotting with anti-Flag antibody. Flag-Nmi was co-precipitated with the full-length HA-BRCA1 (Fig. 1A). In reciprocal experiments of immunoprecipitation with anti-Flag antibody, BRCA1 co-precipitated with Nmi (Fig. 1B).

The in vivo interaction between Nmi and BRCA1 was further
examined in T47D breast cancer cells. Anti-BRCA1 or anti-Nmi immunoprecipitates of extracts from T47D cells and MCF-7 cells, independently performed with two BRCA1 monoclonal antibodies and two Nmi polyclonal antibodies, co-precipitated BRCA1 and Nmi. Anti-BRCA1 monoclonal antibody, but not isotype control antibody, specifically immunoprecipitated the 220-kDa BRCA1 in T47D cells (Fig. 1C). Furthermore, anti-Nmi antibody, but not the control antibody, resulted in the co-immunoprecipitation of endogenous Nmi (Fig. 1C). BRCA1 was reciprocally co-immunoprecipitated with anti-Nmi antibody (Fig. 1D). Similar data were obtained in MCF-7 breast cancer cells and MCF-10A normal breast epithelial cells (data not shown). In addition, immunofluorescence analysis using BRCA1 monoclonal antibody revealed BRCA1 localization in nuclear dots as reported previously (31). In the majority of T47D, MCF-7, and MDA-MB-231 cells, Nmi and BRCA1 are co-localized with one another in nuclear foci (Fig. 1E). The co-immunoprecipitation data and immunostaining results suggest that BRCA1 and Nmi interact directly in vivo.

To map the regions required for BRCA1 interaction with Nmi, a series of GST-BRCA1 deletion mutants were constructed and precipitated with the total cell lysates of 293T cells transfected with Flag-Nmi. As shown in Fig. 2A, Nmi binds to two potential major domains in the BRCA1 protein corresponding to amino acid residues 298–683 and 1301–1863, respectively. In addition, when 293T cells were co-transfected with Flag-Nmi and an HA-BRCA1 cDNA construct containing 1301–1863 aa, the binding of Flag-Nmi with BRCA1 (1301–1863 aa) was observed (Fig. 2B). When 293T cells were cotransfected with Flag-Nmi and an HA-BRCA1 (1–683 aa) construct, the association of Flag-Nmi and HA-BRCA1 (1–683 aa) was also observed (Fig. 2C). These results indicate that the binding of Nmi to BRCA1 is mediated through two BRCA1 domains containing 1301–1863- and 1–683-aa residues.

**Homodimerization of Nmi Enhances Its Association with BRCA1**—Nmi shares 25% amino acid identity with the IFN-γ...
inducible protein IFP 35, which was identified as an IFN-β- inducible gene in HeLa cells (38). The homologous region (90–92 aa) between Nmi and IFP 35 is a novel Nmi/IFP 35 domain repeated in tandem in both proteins. This domain mediates Nmi-Nmi and Nmi-IFP 35 interactions as well as subcellular localization (35). To analyze whether Nmi forms homodimers, two constructs (HA epitope-tagged Nmi and a Flag epitope-tagged Nmi) were used for the co-transfection assay in 293T cells. Immunoprecipitates using anti-Flag antibody were resolved by SDS-PAGE and analyzed by Western blotting using anti-HA antibody. HA-Nmi was observed to bind to Flag-Nmi (Fig. 3A). Similar data were obtained when the samples were immunoprecipitated with anti-HA antibody and analyzed by anti-Flag antibody (Fig. 3A). These results indicate that Nmi can homodimerize in vitro, as reported previously (35).

Next, to elucidate the effects of Nmi homodimerization on BRCA1 interaction with Nmi, 293T cells were co-transfected with Flag-Nmi, HA-BRCA1, and increasing amounts of HA-Nmi. The samples were immunoprecipitated with anti-Flag antibody and subjected to SDS-PAGE and Western blotting with anti-HA antibody. A dose-dependent increase in the association of HA-Nmi with HA-BRCA1 and Flag-Nmi was observed in the presence of increasing amounts of HA-Nmi (Fig. 3B), suggesting that Nmi homodimerization is involved in BRCA1 and Nmi interaction.

BRCA1, Nmi, and c-Myc Are Present in a Complex In Vitro and in Vivo—Nmi has been shown to interact with c-Myc (32), although the biological role of Nmi binding to c-Myc is not well defined. BRCA1 was reported to associate with c-Myc (16). Because both Nmi and BRCA1 bind to c-Myc, we investigated the potential association of Nmi and BRCA1 with c-Myc and the formation of a BRCA1-c-Myc-Nmi complex. 293T cells were co-transfected with HA-BRCA1 and/or pEBG-c-Myc. Total cell lysates were prepared and precipitated with GST-beads. The precipitates were then separated by SDS-PAGE and detected by Western blotting using anti-Flag antibody. Aliquots of these total lysates were also analyzed for expression of Nmi and BRCA1 by Western blotting.

BRCA1 Promoter Activity in Breast Cancer

![Diagram](image) Fig. 4. c-Myc interacts with HA-BRCA1 and Flag-Nmi in 293T cells. A and B, 293T cells were transfected with expression vectors for pEBG-c-Myc (0.5 μg), HA-BRCA1 (0.5 μg), and Flag-Nmi (0.5 μg). Total cell lysates were prepared and precipitated with GST-beads. The precipitates were separated by SDS-PAGE and immunoblotted with anti-HA or anti-Flag antibodies. In addition, aliquots of these total cell lysates were analyzed for expression of HA-BRCA1 and Flag-Nmi by Western blotting (WB). C, Flag-Nmi increases c-Myc and HA-BRCA1 interaction in 293T cells. 293T cells were co-transfected with HA-BRCA1 (0.375 μg), pEBG-c-Myc (0 or 0.5 μg), and increasing amounts of Flag-Nmi cDNA construct as indicated. After 48 h, total cell lysates were prepared and precipitated with GST-beads. The complexes were then separated by SDS-PAGE and detected by Western blotting using anti-Flag antibody. Aliquots of these total lysates were also analyzed for expression of Nmi and BRCA1 by Western blotting.
Nmi, and pRc-c-Myc expression constructs, c-Myc increased the interaction of BRCA1 and Nmi in T47D cells (Fig. 5A) as well as in 293T cells (data not shown), indicating that breast cancer cells expressed the tricomplex of Nmi/BRCA1/c-Myc.

We next investigated the presence of endogenous c-Myc/BRCA1-Nmi complexes in T47D breast cancer cells and in MCF-10A normal breast epithelial cells. Cells were cultured in low serum (5%) or high serum (20%) medium as indicated, and total cell lysates were immunoprecipitated (IP) with anti-Flag antibody. The immunoprecipitates were analyzed by Western blotting using anti-Nmi antibody. In addition, aliquots of these lysates were analyzed for expression of Nmi, c-Myc, and BRCA1 as indicated by Western blotting. B and C, endogenous c-Myc/BRCA1-Nmi complex in T47D cells and MCF-10A cells. 1 × 10⁷ cells were grown in 5% or 20% fetal bovine serum medium overnight and then lysed. Cell lysates were centrifuged for 15 min at 4°C. Supernatants were preincubated for 2 h at 4°C with protein G-Sepharose beads and normal rabbit IgG (C lane). Immunoprecipitations were performed for 4 h at 4°C using anti-Nmi rabbit polyclonal antibody (R1445). Immunoprecipitates were then subjected to Western blotting for c-Myc detection using c-Myc monoclonal antibody (9E10) and for BRCA1 detection using BRCA1 monoclonal antibody (H945.2), respectively. The blots were stripped and reprobed with anti-Nmi antibody (R1373). D, Nmi and c-Myc co-localized in the nucleus of MDA-MB-231 cells. MDA-MB-231 cells were transiently transfected with c-Myc construct. Cells were cultured for 2 days, and immunostained dually with Nmi polyclonal antibodies (green) and c-Myc monoclonal antibodies (red). Significant co-localization of Nmi and c-Myc in the nucleus was observed as indicated by the presence of the yellow color in the merged image.

Nmi, and pRc-c-Myc expression constructs, c-Myc increased the interaction of BRCA1 and Nmi in T47D cells (Fig. 5A) as well as in 293T cells (data not shown), indicating that breast cancer cells expressed the tricomplex of Nmi/BRCA1/c-Myc.

We next investigated the presence of endogenous c-Myc/BRCA1-Nmi complexes in T47D breast cancer cells and in MCF-10A normal breast epithelial cells. Cells were cultured in low serum (5%) or high serum (20%) medium as indicated, and total cell lysates were immunoprecipitated with anti-Nmi antibody (R1445) and anti-c-Myc antibody. As shown in Fig. 5, B and C, in the presence of low serum, we observed Nmi and c-Myc association, whereas no association of Nmi with BRCA1 was observed. However, in the presence of high serum, we observed an endogenous association of Nmi with BRCA1, and the association between Nmi and c-Myc was also significantly increased (Fig. 5, B and C). To address Nmi and c-Myc nuclear co-localization, we transfected MDA-MB-231 cells with the c-Myc expression plasmid, pRc-c-Myc, followed by immunofluorescence analysis with anti-c-Myc monoclonal antibody (9E10) and anti-Nmi rabbit polyclonal antibody (R1445). As shown in Fig. 5D, nuclear staining of c-Myc (red) and endogenous Nmi (green), as well as co-localization of c-Myc and Nmi (yellow) were observed. These data indicate that Nmi, BRCA1, and c-Myc can form a complex under growth conditions and that Nmi functions as an adaptor molecule to recruit c-Myc with BRCA1.

BRCA1 and Nmi Down-regulate c-Myc-induced hTERT Promoter Activity—Telomerase maintenance has been proposed as an essential prerequisite to human tumor development. The telomerase reverse transcriptase component (TERT) is not expressed in most primary human cells and tissues but is up-regulated in the majority of immortalized cell lines and tumors (28–30, 39). c-Myc induces telomerase activity in human mammary epithelial cells and normal human diploid fibroblasts (23, 25, 33). Because Nmi, BRCA1, and c-Myc form a complex, we investigated the effects of BRCA1 and Nmi on c-Myc-induced human hTERT promoter activity in breast cancer cells. T47D breast cancer cells were co-transfected with Flag-Nmi, pRc-c-
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Myc, HA-BRCA1, and a reporter plasmid containing the core promoter of hTERT (pGL3 vector inserted with the 181-bp promoter region of hTERT) as described (33, 40), and the activity of the hTERT promoter was measured using a luciferase assay kit (Promega). Significant activation of the hTERT promoter in the presence of c-Myc was observed as expected in 293T cells (data not shown) as well as in T47D cells (Fig. 6, A and B), whereas no significant change in the basal activity of the hTERT promoter was observed with the introduction of Flag-Nmi or HA-BRCA1 alone. Furthermore, in T47D cells, HA-BRCA1 or Flag-Nmi alone had no significant effect on c-Myc-induced hTERT promoter activity (Fig. 6A), whereas c-Myc alone activated the hTERT promoter about 6-fold. c-Myc-induced hTERT promoter activity was blocked (~75% inhibition) by the combination of Flag-Nmi and HA-BRCA1. Importantly, upon removal of Flag-Nmi, HA-BRCA1 failed to inhibit c-Myc-induced hTERT promoter activity (Fig. 6A).

Two BRCA1 mutants identified in familial breast cancers, the missense mutation A1708E and the nonsense mutation Y1853X, bound c-Myc or Nmi but failed to reduce c-Myc-induced hTERT promoter activity in T47D cells (Fig. 6, D, E, and C, respectively). These results demonstrate that c-Myc led to significant activation of hTERT transcription, whereas Nmi together with wild-type BRCA1 significantly blocked hTERT promoter activity induced by c-Myc. However, Nmi together with mutated BRCA1 failed to block c-Myc-induced hTERT promoter activity, indicating that the BRCT domain of BRCA1 is important for its interaction with Nmi and in regulating the hTERT promoter activity induced by c-Myc.

We further investigated the effects of Nmi and BRCA1 on hTERT promoter activity using the hormone inducible form of c-Myc in stable transfected NIH 3T3/c-Myc-ER cells (24). Upon treatment of these cells with 4-hydroxytamoxifen (4-OHT), c-Myc expression and activity are induced, resulting in c-Myc-enhanced hTERT promoter activity (24). As shown in Fig. 7, treatment of NIH 3T3/c-Myc-ER cells with 0.75 μM 4-OHT induced hTERT promoter activity by ~5-fold. This induction was completely abolished in the presence of BRCA1 together with Nmi, whereas BRCA1 or Nmi alone had no effect on 4-OHT-induced hTERT promoter activity. Furthermore, mutated BRCA1 failed to inhibit hTERT promoter activity in the presence of 4-OHT and Nmi, indicating the involvement of intact BRCA1 in regulating hTERT promoter activity in the presence of Nmi. Thus, BRCA1 regulates c-Myc-induced hTERT promoter activity in the presence of Nmi.

**Fig. 6.** Nmi and BRCA1 inhibit c-Myc-induced hTERT promoter activity. A, T47D cells were transfected with the hTERT promoter reporter plasmid pGL3-hTERT (181 bp of the hTERT core promoter) in combination with pRc-c-Myc, wild-type HA-BRCA1, and Flag-Nmi expression plasmids as indicated. pCMVβ-gal and pcDNA3 vectors were also included for normalization of the transfection. Cells were assayed for luciferase and β-galactosidase activities after 48 h. The expression of luciferase from the hTERT promoter was analyzed using a luciferase assay kit (Promega). Luciferase measurements, made using 40 μl of cell lysate, are presented as the mean ± S.D. of the fold activation compared with the control vector. Each experiment was performed at least three times in triplicate. B, 30 μg of total cell lysates from the transfected cells were prepared and were analyzed for c-Myc expression level by Western blot (WB) analysis. C, T47D cells were transfected with pGL3-hTERT (181-bp hTERT promoter) in combination with Flag-Nmi, pRc-c-Myc, wild-type HA-BRCA1, or mutant HA-BRCA1 (A1708E or Y1853X) expression plasmids as indicated. pCMVβ-gal was also included for normalization of the transfection. 48 h after the transfection, luciferase activity was analyzed using a luciferase assay kit (Promega). The data are presented as the mean ± S.D. of four independent experiments performed in triplicate. D, c-Myc interacts with mutated and wild-type BRCA1. 293T cells were transfected with the expression vectors for either wild-type HA-BRCA1 (0.5 μg) or mutated BRCA1 (A1708E and Y1853X), and pEBG-c-Myc (0.5 μg). Total cell lysates were prepared and precipitated with GST-beads. The precipitates were separated by SDS-PAGE and immunoblotted with anti-Flag antibody. E, Nmi interacts with mutated and wild-type BRCA1. 293T cells were transfected with the expression vectors for either wild-type HA-BRCA1 (0.5 μg) or mutated BRCA1 (A1708E and Y1853X) and Flag-Nmi (0.375 μg). Total cell lysates were prepared and immunoprecipitated with anti-Flag antibody. The immunoprecipitates were separated by SDS-PAGE and immunoblotted with anti-HA antibody.
BRCA1 loss-of-function germ-line mutations in the tumor-suppressor instead cause genetic instability, subjecting cells to a high risk fails to suppress c-Myc-induced promoter activity in breast cancer cells. Mutated BRCA1 We have shown a novel association of BRCA1 with Nmi and c-Myc. This complex may function as a tumor novel role as a component of a transcription factor complex that and Nmi can form a complex with c-Myc in vivo which BRCA1 acts as a tumor suppressor might be through the

FIG. 7. Effects of Nmi and BRCA1 on hTERT promoter activity in NIH 3T3/c-Myc-ER cells. NIH 3T3/c-Myc-ER cells were transfected with 0.5 μg of hTERT reporter plasmid, 0.5 μg of HA-BRCA1, and 0.25 μg of Flag-Nmi as indicated. 0.5 μg of pCMVβ-gal was used to determine the transfection efficiency. After 6 h of transfection, 0.75 μM of 4-OHT was added to the cells to induce a c-Myc-ER fusion protein. At 48 h after transfection, a luciferase activity assay was performed, and luciferase activities were normalized by β-galactosidase activity. The results represent three independent experiments, and every experiment was done in triplicate. WT, wild type.

DISCUSSION

Hereditary breast and ovarian cancers can be caused by loss-of-function germ-line mutations in the tumor-suppressor gene BRCA1 (1–3). Current evidence demonstrates that mutations in BRCA1 do not directly result in tumor formation, but instead cause genetic instability, subjecting cells to a high risk of malignant transformation. BRCA1 is in a class of caretaker genes, which function in maintaining genetic stability (1–3).

We have shown a novel association of BRCA1 with Nmi in vitro and in vivo and have observed that Nmi homodimerization enhances Nmi association with BRCA1. Furthermore, BRCA1 and Nmi can form a complex with c-Myc in vitro and in vivo. This tricomplex results in inhibition of c-Myc-induced hTERT promoter activity in breast cancer cells. Mutated BRCA1 (A1708E and Y1853X) also associates with Nmi and c-Myc but fails to suppress c-Myc-induced hTERT promoter activity. Therefore, our results strongly suggest that BRCA1 can play a novel role as a component of a transcription factor complex that contains Nmi and c-Myc. This complex may function as a tumor suppressor by regulating c-Myc-induced hTERT promoter activity.

c-Myc is an important regulator of many cellular processes, including growth promotion, differentiation, and apoptosis. However, the mechanisms underlying c-Myc biological activity remain elusive. The C-terminal domain of c-Myc mediates its interaction with Max and physiological recognition of DNA target sequences, events needed for all biological actions. Interactions between the C-terminal domain and other cellular proteins, including YY-1, AP-2, BRCA1, TFI-I, and Miz-1, suggest levels of regulatory complexity beyond Max in controlling DNA recognition by c-Myc (21–22, 26). An intact HLH region was required in c-Myc for efficient association with BRCA1 (16). BRCA1 does not, however, bind to Max (16). BRCA1-repressed c-Myc-mediated transcription adds another level of regulation to the c-Myc network. As observed in our study, BRCA1 and Nmi together down-regulate c-Myc-induced hTERT promoter activity, suggesting that the mechanism by which BRCA1 acts as a tumor suppressor might be through the down-modulation of some targets of c-Myc transcriptional activity.

Based on our findings, Nmi acts as an adaptor and recruits BRCA1 to a complex with c-Myc under cell growth conditions. The presence of the endogenous c-Myc-BRCA1-Nmi complex in T47D and MCF-10A cells under growth conditions suggests that the interaction of these three components plays a role during proliferation, probably by inhibiting the c-Myc-induced proliferation of breast cancer cells. Consistent with its association in cells, overexpression of BRCA1 selectively repressed c-Myc-mediated transactivation and inhibited Ras co-transformation of embryonic fibroblasts by c-Myc but not by SV40T antigen. Thus, BRCA1 and Nmi may act in part by regulating the oncogenic potential of c-Myc.

Because c-Myc can elevate telomerase activity in normal epithelial cells and fibroblasts to a level approximating that observed in tumor cell lines, increased c-Myc activity could account for the presence of telomerase in many late-stage tumors (28–30). Although the c-myc oncogene may induce telomerase activity in a significant proportion of tumors, telomerase may also be regulated by other pathways that contribute to transformation (28–30). Interestingly, based on our observations, mutated BRCA1 alone or in the presence of Nmi had no effect on c-Myc-induced hTERT promoter activity. Thus, mutations in BRCA1 associated with hereditary breast cancer interfere with the suppression of c-Myc-induced hTERT promoter activity, leading to the sustained activation of telomerase, a prerequisite in carcinogenesis. Taken together, these findings demonstrate a new cellular function of BRCA1 and provide insight into the molecular mechanisms involved in the development of breast cancer.

Acknowledgments—We are very thankful to Drs. Jerome E. Groopman, David Fisher, and Shalom Avraham for critical review of the manuscript; Mikiung Kim-Park and Sarah Evans for typing the manuscript; Dan Kelley for help with preparation of the figures; and Janet Delahanty for editing the manuscript. We thank Dr. Tina Kuus-Reichel for the BRCA1 monoclonal antibodies, Dr. R. Scully for the BRCA1 expression construct, Dr. A. S. Zervos (32) for the HA-Nmi construct, and Dr. S. Kyo (33) for the pGL3-hTERT (181 bp).

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