Increased Expression of the E3 Ubiquitin Ligase RNF5 Is Associated with Decreased Survival in Breast Cancer

Kenneth D. Bromberg, Harriet M. Kluger, Agnes Delaunay, Sabihia Abbas, Kyle A. DiVito, Stan Krajewski, and Ze’ev Ronai

Abstract
The selective ubiquitination of proteins by ubiquitin E3 ligases plays an important regulatory role in control of cell differentiation, growth, and transformation and their dysregulation is often associated with pathologic outcomes, including tumorigenesis. RNF5 is an E3 ubiquitin ligase that has been implicated in motility and endoplasmic reticulum stress response. Here, we show that RNF5 expression is upregulated in breast cancer tumors and related cell lines. Elevated expression of RNF5 was seen in breast cancer cell lines that became more sensitive to cytochalasin D– or paclitaxel-induced apoptosis following its knockdown with specific short interfering RNA. Inhibition of RNF5 expression markedly decreased cell proliferation and caused a reorganization of the actin cytoskeleton in response to stress in MCF-7 but not in p53 mutant breast cancer cells, suggesting a p53-dependent function. Significantly, high levels of RNF5 were associated with decreased survival in human breast cancer specimens. Similarly, RNF5 levels were higher in metastatic melanoma specimens and in melanoma, leukemia, ovarian, and renal tumor-derived cell lines, suggesting that increased RNF5 expression may be a common event during tumor progression. These results indicate that RNF5 is a novel regulator of breast cancer progression through its effect on actin cytoskeletal alterations, which also affect sensitivity of breast cancer cells to cytoskeletal-targeting antineoplastic agents.

Introduction
Ubiquitin E3 ligases regulate a vast number of cellular processes, including development, proliferation, cell cycle control, transcription, apoptosis, and DNA repair (1–3). It is evident that many really interesting new gene (RING) finger proteins, which have been implicated in cell growth and development, possess E3 ligase activity and mediate the ubiquitination of their substrates (4). A growing number of RING E3 ligases are aberrantly expressed or regulated during tumorigenesis (4, 5). Depending on the specific substrate, an E3 ligase can either promote or inhibit cancer development. For example, the MDM2 oncogene, which targets p53 for ubiquitination-dependent degradation, is overexpressed in human tumors (6). In contrast, the tumor suppressors BRCA1 and BRCA2 and von Hippel-Lindau are frequently mutated or deleted in human tumors (7, 8). RNF5 is an 18-kDa RING finger E3 ligase that is important for development in Caenorhabditis elegans by regulating proper formation of muscle attachment sites (9). In mammalian cells, RNF5 regulates cell motility by ubiquitinating the focal adhesion protein paxillin (10), which triggers the exclusion of paxillin from the focal adhesions. RNF5 (a.k.a. RMA1) was also shown to serve as a quality control protein in the endoplasmic reticulum, through which it contributes to the clearance of malfolded proteins, including mutant CFTR (∆I08; ref. 11). RNF5/RMA1 was also shown to affect the Salmonella protein SOP-A by ubiquitination that facilitates its trafficking from the endosomes/vacuoles to the cytosol (12), further substantiating the role of this ligase in control of protein localization in the cell. In this study, we show that RNF5 modulates breast cancer cell sensitivity to cytoskeletal-targeting anticancer agents and that RNF5 expression inversely correlates with survival of breast cancer patients. Our findings suggest that through the dynamic rearrangement of the actin cytoskeleton and increased cell proliferation, RNF5 plays an important role in neoplastic progression of breast cancer and possibly other human tumors.

Materials and Methods

Tumor tissue mRNA array. The BioChain Human Adult Tumor/Normal Tissues mRNA Array (BioChain Institute, Inc.) was used to analyze RNF5 expression. This array contains equal amounts of mRNA from 47 tumor tissues and their corresponding normal tissues. Biotinylated DNA probes recognizing either RNF5 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts were generated by random labeling using the NEBlot Phototope kit (New England Biolabs). Hybridization was carried out overnight using FastHyb-Hybridization Solution (BioChain Institute) according to the manufacturer's instructions. Levels of RNF5 or GAPDH transcripts were generated by random labeling using the NEBlot Phototope kit (New England Biolabs). Hybridization was carried out overnight using FastHyb-Hybridization Solution (BioChain Institute) according to the manufacturer's instructions. Levels of RNF5 or GAPDH were detected by enhanced chemiluminescence (ECL) using the Phototope-Star detection kit (New England Biolabs) and analyzed on a Bio-Rad GS800 scanning densitometer. The RNF5 expression level for each tissue and its corresponding normal tissue was normalized to GAPDH and then compared.

Cell lines and cell culture. Breast carcinoma MCF-7, SKBR3, BT-24, BT-474, MDA-MB-231, MDA-MB-435, and ZR-75-1 cells were cultured in growth medium [DMEM and 10% fetal bovine serum (FBS) and supplemented with 100 units penicillin/mL and 100 units streptomycin/mL]. Human mammary epithelial cells (HMEC) were cultured in MEGM mammary epithelial cell medium supplemented with singlequots (Clonetics).

RNA isolation and reverse transcription-PCR analysis. Total RNA was purified with the Qiagen RNeasy kit according to the manufacturer’s instructions. cDNA synthesis was done with the Moloney murine leukemia
virus reverse transcriptase (Invitrogen) using 2 μg RNA and a polydeoxynucleotide as a template. Semiquantitative PCR was carried out for 20 cycles using [α-32P]dCTP (Amersham Biosciences) and 10 ng cDNA. Reaction products were resolved by 5% PAGE and visualized and quantified on a Bio-Rad FX Molecular Imager.

**Western blot.** Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (PBS, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 1 mmol/L EDTA) supplemented with protease and phosphatase inhibitors. Western blotting was done using standard procedures with antibodies directed against RNF5 and visualized by ECL. Antibodies directed against β-actin and α-tubulin were used as loading controls.

**National Cancer Institute 60-tumor cell line panel microarray.** Established tumor cell lines were cultured as described previously (13), harvested after fixation, and embedded in paraffin to be spotted onto a tissue microarray block for immunohistochemical screening. The cell lines included the National Cancer Institute (NCI) 60-tumor cell line panel (14).

**RNF5 short interfering RNA and retroviral infections.** The pSUPER retro vector was used to introduce short interfering RNA (siRNA) targeting RNF5 and its corresponding scramble control into MCF-7, MDA-MB-231, MDA-MB-435, and BT-474 cells. pSUPER.retro.shRnf5 or pSUPER.retro scramble infectious retroviral stock was generated in human 293T cells. The above indicated cells were infected for 6 h with the addition of 5 μg/mL polybrene. After 48 h, cells stably expressing RNF5 siRNA were selected by treatment with 2 μg/mL puromycin. Alternatively, MCF-7 cells were transfected with an oligonucleotide siRNA targeting RNF5 or a scramble siRNA (Ambion) using LipofectAMINE 2000 (Invitrogen) in Opti-MEM.

**Figure 1.** Increased RNF5 expression in human cancers. A, mRNA array of 47 tumors and their corresponding normal tissues. The array was probed for RNF5, stripped, and then reprobed for GAPDH. Tumor mRNA is spotted in duplicate in odd-numbered rows (T) and mRNA from the corresponding normal tissues is spotted to the right of the tumor in the even-numbered rows (N). Lane 1, positive control; lane 2, negative control. B, tumors that displayed greater than a 2.5-fold increase in RNF5 expression compared with normal tissue. The colors of the rectangles enclosing the spots quantified in (A) correspond to the matching colors representing RNF5 expression levels in (B). C, increased RNF5 mRNA expression in breast carcinoma cell lines. RNA was isolated from the indicated cell lines and semiquantitative RT-PCR was done with primers specific for RNF5 or GAPDH. Left, representative gel of three independent experiments; right, quantification. D, increased RNF5 protein expression in breast carcinoma cell lines. The indicated cell lines were lysed in RIPA buffer and the level of endogenous RNF5 in the whole-cell lysates was analyzed by Western blot. β-Actin was used as a loading control.
Three oligonucleotides targeting different sequences in the \textit{RNF5} gene were used. The medium was changed 6 h after transfection and experiments were done 48 h after transfection.

\textbf{Fluorescence-activated cell sorting cell cycle analysis.} Cells at approximately 50\% to 60\% confluence were treated for 24 h with DMSO, 5 \textmu\text{M} cytochalasin D, or 1 \textmu\text{M} paclitaxel (Sigma) for 24 h. Cells were harvested, washed twice in PBS, and fixed in ice-cold 70\% ethanol overnight at 4°C. Cells were washed twice in PBS and then labeled with propidium iodide solution (50 \textmu\text{g/mL} propidium iodide, 10 \textmu\text{g/mL} RNase A, 0.1\% sodium citrate, 0.1\% Triton X-100) for 30 min at room temperature. Fluorescent-stained cells were then transferred to polystyrene tubes with cell strainer caps (BD Falcon) and subjected to fluorescence-activated cell sorting using a Becton Dickinson FACScan. Acquisition and analysis of cells containing sub-G1 DNA content (apoptotic cells) were done using CellQuest 3.2 software (Becton Dickinson).

\textbf{Cell proliferation.} MCF-7 cells were plated in 96-well plates at a density of 5,000 cells per well. After an overnight incubation, cells were treated with 20 \textmu\text{L} CellTiter 96 Aqueous One Solution Reagent (Promega). The cells were further incubated at 37°C for 1 h and cell growth was quantified at an absorbance of 480 nm on an Envision 2101 multilabel reader (Perkin-Elmer).

\textbf{Confocal microscopy.} MCF-7 cells were grown on glass coverslips, fixed, and permeabilized according to the protocol of Didier et al. (10). After blocking in 5\% bovine serum albumin for 30 min, cells were incubated with 100 \textmu\text{L} of monoclonal antibodies directed against paxillin (Upstate) or \textalpha\-tubulin (Sigma) for 1 h at room temperature followed by washing and further incubation with a goat anti-mouse Alexa Fluor 568–conjugated secondary antibody and Alex Fluor 488–phalloidin (Invitrogen) for 1 h. The coverslips were mounted on glass slides in ProLong Gold (Invitrogen) and images were acquired on a Zeiss LSM 510 Meta confocal microscope at \times 63 magnification.

\textbf{Tissue microarrays.} The breast cancer and melanoma tissue microarrays were constructed as described previously (18). Immunohistochemical staining of the arrays with polyclonal antibodies against RNF5 was carried according to the protocol of Chung et al. (19). The regions of most intense staining were scored by eye for each spot. The staining was graded using the following scale: 0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining. See Supplementary Materials and Methods for tissue microarray construction and details.

\section*{Results}

\textbf{RNF5 expression is elevated in human cancers.} A growing number of ubiquitin ligases that are important regulators of development and cell growth are found to be aberrantly expressed in human tumors. To evaluate the expression pattern of RNF5 in human tumors, we used a mRNA array that contained mRNA from 47 different tumors and their corresponding normal tissues...
(Fig. 1A). For each tumor, the mRNA was spotted in duplicate. After probing the array for RNF5 expression, the two duplicate spots were averaged and then normalized to the expression levels of the housekeeping gene GAPDH. As seen in Fig. 1B, seven different tumors displayed at least a 2.5-fold increase in RNF5 mRNA levels. RNF5 expression was markedly increased in several breast cancers, with an ~10-fold increase in breast intraductal carcinoma and an ~3-fold increase in breast adenocarcinoma. Marked increases in RNF5 expression were also observed in lung cancer (~5-fold) and esophageal cancer (~4-fold).

Next, we sought to confirm the results of the mRNA array by examining RNF5 expression in breast cancer cell lines by reverse transcription-PCR (RT-PCR) analysis. As shown in Fig. 1C, RNF5 mRNA levels were increased ~3.5-fold in several breast cancer cell lines compared with HMECs, including MCF-7, BT-20, and BT-474 cells. These results were confirmed on the protein level via Western blotting with antibodies directed against endogenous RNF5 (Fig. 1D). To confirm RNF5 protein expression in other cancers, we generated and probed a cell microarray of the NCI 60-tumor cell line panel. Consistent with the mRNA tumor array, strong RNF5 expression was seen in breast, colon, and lung cancer cell lines (Fig. 2). In addition, RNF5 was strongly expressed in several ovarian, renal, melanoma, and leukemia cell lines. Taken together, these findings indicate that RNF5 is strongly expressed in many different cancers and that RNF5 is up-regulated on the transcriptional level in several human breast cancers.

RNF5 siRNA enhances breast cancer cell apoptosis. To examine the role of RNF5 in breast tumorigenesis, siRNA was used to knock down RNF5 expression in several breast cancer cell lines, including MCF-7, MDA-MB-231, MDA-MB-435, and BT-474 cells. The efficiency of siRNA targeting RNF5 was >80%, which was confirmed in each experiment by Western blot (Fig. 3A and B). Because RNF5 has been shown to regulate cell motility (10), we assessed whether RNF5 altered breast cancer cell migration using a pipette scratch assay. RNF5 siRNA did not have a significant effect on the migratory capacity of any of the cell lines compared with scramble siRNA (Supplementary Fig. S1; data not shown). Consistent with this result, RNF5 knockdown did not affect paxillin localization in MCF-7 cells (data not shown). These data suggest that unlike nontransformed cultures, RNF5 does not affect paxillin or cell migration in the breast cancer cells. Next, we examined whether loss of RNF5 expression altered breast cancer cell death. Knockdown of RNF5 expression did not induce apoptosis in MCF-7, MDA-MB-231, MDA-MB-435, or BT-474 cells (data not shown). However, MCF-7, MDA-MB-231, and BT-474 cells expressing RNF5 siRNA displayed increased apoptosis on treatment with the actin-disrupting agent cytochalasin D compared with scramble-expressing cells (Fig. 3C). A similar enhancement of cytochalasin D–induced apoptosis was observed for MCF-7 cells transfected with three other siRNAs targeting different RNF5 sequences (Supplementary Fig. S2). In addition, MDA-MB-435 cells expressing RNF5 siRNA, which were less affected by cytochalasin, exhibited greater sensitivity to treatment with the microtubule-targeting drug paclitaxel compared with scrambled siRNA-expressing cells (Fig. 3D). RNF5 siRNA also sensitized BT-474 cells, albeit to a lesser degree, to paclitaxel treatment (Fig. 3D). These results indicate that...
RNF5 contributes to the degree of sensitivity of breast cancer cells to cytoskeletal-targeting anticancer agents.

Loss of RNF5 causes rearrangement of the actin cytoskeleton and decreased cell proliferation in MCF-7 cells. Because loss of RNF5 expression sensitized breast cancer cells to cytoskeletal-targeting agents, we examined the actin and microtubule architecture of breast cancer cells expressing scramble or RNF5 siRNA. For MCF-7 cells growing asynchronously, no differences between the two cell lines were observed in the actin cytoskeleton (Fig. 4A) or paxillin (data not shown) localization. However, marked differences in the organization of the actin cytoskeleton were observed after serum starvation. Approximately 55% of scrambled siRNA-expressing MCF-7 cells retained their network of actin fibers (Fig. 4A and B). In contrast, only 21% of RNF5 siRNA-expressing MCF-7 cells were able to maintain their actin cytoskeleton and displayed mainly peripheral actin staining (Fig. 4A and B). After 2-h recovery in serum, both cell types began to elongate back to their original morphology and RNF5 siRNA cells were able to reestablish their actin cytoskeletal architecture. Similar results were observed for MCF-7 cells transfected with three other siRNAs targeting different RNF5 sequences (Supplementary Fig. S3). RNF5 siRNA did not affect MCF-7 cell microtubule cytoskeletal organization under the above conditions (Supplementary Fig. S3; data not shown). Of interest, analysis of cytoskeletal proteins in melanoma cells that were subjected to elevated RNF5 expression also revealed altered pattern of actin expression (Supplementary Fig. S4), further noting the effect on RNF5 on select cytoskeletal proteins. These data suggest that RNF5 contributes to cytoskeletal organization in MCF-7 cells.

To examine the effect of RNF5 on MCF-7 cell proliferation, we used a colorimetric 3-(4-5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay. As shown in Fig. 4C, siRNA of RNF5 markedly decreased MCF-7 cell proliferation compared with scrambled siRNA in both 2% FBS and high (10% FBS) serum. Knockdown of RNF5 in MCF-7 cells transfected with three other siRNAs targeting different RNF5 sequences also decreased cell proliferation (Supplementary Fig. S5).

In contrast to these results in MCF-7 cells, RNF5 siRNA did not affect the actin cytoskeletal architecture or cell proliferation of MDA-MB-231, MDA-MB-435, or BT-474 cells (Supplementary Figs. S6 and S7). However, consistent with the increased apoptosis in MDA-MB-435 cells induced by paclitaxel, MDA-MB-435 cells displayed a reorganization of the microtubule architecture in asynchronously growing cells (Supplementary Fig. S6B). No differences
in microtubule architecture were observed for MDA-MB-231 or BT-474 cells (Supplementary Fig. S6A and S6C). The differences between cell lines may be due to the fact that MCF-7 cells express wild-type p53 and MDA-MB-231, MDA-MB-435, or BT-474 cells either have mutations in or do not express p53 (20, 21).

To test the possibility that functional p53 is required for RNF5-dependent effects on cytoskeletal architecture, we have inactivated p53 in the MCF-7 cells and tested whether the changes seen above would be attenuated. To this end, we have expressed the human papilloma virus E6 protein, which is among the potent ligases for p53. Knockdown of RNF5 in MCF-7 caused an increase in p53 levels and expression of E6 in these cells caused efficient decrease in p53 levels (Fig. 5A). Decrease of p53 levels in the MCF-7 cells ameliorated the effects of RNF5 siRNA on MCF-7 cell proliferation and actin cytoskeletal architecture (Fig. 5B and C). These data suggest that RNF5 contributes to cytoskeletal organization and affects cell proliferation in part via functional p53.

**Decreased survival in breast cancers expressing RNF5.** To further investigate the significance of dysregulated RNF5 in human cancers, we evaluated RNF5 expression in human breast cancer specimens. Of the 655 breast cancer tumors on the tissue microarrays, 479 (73%) were interpretable for cytoplasmic RNF5.

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**Figure 5.** Inhibition of p53 attenuates the decrease in cell proliferation and actin cytoskeleton reorganization in response to knockdown of RNF5 expression in MCF-7 cells. A, MCF-7 cells stably expressing scramble siRNA (SC) or RNF5 siRNA were transfected with human papilloma virus E6 protein (E6) or pCDNA3 as a control (Con). Cells were lysed in RIPA buffer and the level of p53 in the whole-cell lysates was analyzed by Western blot with a p53 antibody. α-Tubulin was used as a loading control. B, MCF-7 cells stably infected with scramble (SC) or RNF5 siRNA were transfected with human papilloma virus E6 protein (E6) or pCDNA3 as a control (Con). Cell proliferation was measured as described in Materials and Methods. Each experiment was done in triplicate. Error bars, SE of three sets of experiments. *, P < 0.03, difference between pCDNA3- and E6-transfected RNF5 siRNA-expressing MCF-7 cells for cell proliferation. C, representative confocal microscopic images showing the cellular localization of actin and α-tubulin in MCF-7 cells stably expressing scramble siRNA or RNF5 siRNA that were transfected with pCDNA3 or E6. Cells were processed as per Fig. 4A.
staining. Spots that were deemed uninterpretable had insufficient tumor cells in the spot, loss of tissue in the spot, or an abundance of necrotic tissue. Examples of scores of positive and negative spots of necrotic tissue. Survival information was available for 424 (90%) tumor cells in the spot, loss of tissue in the spot, or an abundance of necrotic tissue. Staining was further examined. RNF5 staining was associated with decreased survival (P = 0.017). No association was found with survival or other clinical variables.

**Discussion**

In the current study, we show that the RING finger E3 ligase RNF5 is up-regulated on the transcription level in breast cancer and that loss of RNF5 expression sensitizes breast cancer cells to cytochalasin D– and paclitaxel-induced apoptosis. RNF5 siRNA also causes a reorganization of the actin cytoskeleton in response to stress and a marked inhibition of cell proliferation in MCF-7 cells. Moreover, increased RNF5 expression in human breast cancer specimens is associated with decreased survival. It is of interest to note that noninvasive breast cancer (intraductal) has higher RNF5 expression than invasive breast cancer, suggesting changes in RNF5 expression (and likely activity) during select stages of breast cancer development. These results suggest that RNF5 is a novel regulator of breast cancer development and suggest that increased expression of RNF5 may contribute to breast tumor progression.

Previously, we have shown that expression of RNF5 in non-transformed mouse fibroblast NIH-3T3 cells excludes paxillin from the focal adhesions and inhibits cell migration (10). Here, we show that RNF5 can modulate breast cancer apoptosis and plays a role in promoting breast cancer progression. Whereas RNF5 is involved in organization of the actin cytoskeleton, it does not seem to affect cell migration or paxillin localization in the breast cancer cell lines. Thus, RNF5 substrate(s) in the breast cancer and possibly other tumor cells are different than those identified in the nontransformed cells. The latter could be explained by different post-translational modification of paxillin in the tumor cells or the modification and activity of RNF5, aspects that deserve further studies. In addition to the tumors with increased RNF5 expression (Fig. 1B), we have identified several other tumors with decreased RNF5 expression, which we are presently studying. In this manner, RNF5 is expected to affect different cellular targets, which play an important role in cytoskeletal organization and function. Because RNF5 was also implicated in the clearance of malfolded proteins, it is likely that RNF5 affects other targets in breast cancer cells than those studied thus far.

It is important to delineate the mechanisms by which RNF5 modulates drug-induced apoptosis and regulates the actin cytoskeleton and cell proliferation to promote tumor progression. To this end, RNF5 may function somewhat similar to TRIM 32, which has been shown to interact with myosin and to ubiquitinate actin (22). Alternatively, RNF5 activity may affect Rho family of GTPases, which are among key regulators of actin and microtubule cytoskeleton (23) and which are often overexpressed or hyperactive in human breast cancers (24). Along these lines, the ubiquitin E3 ligase Smurf1 targets RhoA for degradation at lamellipodia and filopodia and is required for the morphology of transformed 293T cells (25).

The tyrosine kinase Src also can alter cell morphology and the actin cytoskeleton during tumorigenesis. A previous study has shown that the ubiquitin E3 ligase Hakai ubiquitinates E-cadherin and disrupts cell-cell contacts in epithelial cells in response to Src activation (26). A recent study has also indicated that Src can modulate sensitivity to paclitaxel-induced cytotoxicity by phosphorylating caveolin-1 (27). Whether RNF5 is a regulator or an effector of a Rho GTPase or a Src signaling network that reorganizes...
cytoskeletal architecture and enhances cell proliferation during tumorigenesis remains to be determined.

The loss of RNF5 expression in MCF-7 cells led to a decrease in cell proliferation and alterations in actin cytoskeleton in response to stress but did not affect in either MDA-MB-231, MDA-MB-435, or BT-474 cells. This may be attributable to the differences in p53 status in these cell lines, as only MCF-7 cells express a functional p53 (20, 21). Further supporting this hypothesis was the finding that reducing p53 levels in MCF-7 cells by the expression of E6 attenuated the effects of RNF5 siRNA on actin cytoskeleton and cell proliferation. MCF-7 cells were also the most sensitized cell line to cytochalasin D–induced apoptosis of the RNF5 siRNA-expressing cells. Similar to this effect in breast cancer, p53 status can modulate the sensitivity of non–small cell lung cancer cells to gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor (28). RNF5 may signal through the above-mentioned Rho GTPase or Src networks or ERAD to inhibit p53, as loss of RNF5 expression also increased p53 expression in MCF-7 cells (Fig. 5A).

Because RNF5 was strongly expressed in several different cancers and RNF5 levels were higher in metastatic melanoma specimens than primary tumors, increased RNF5 expression may be a common event during tumor progression. Given its role in modulating anticancer drug-induced apoptosis and its association with clinical prognosis, RNF5 should be further assessed as a marker and possible drug target in breast cancer.

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References

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