

# Suppression of the Negative Regulator LRIG1 Contributes to ErbB2 Overexpression in Breast Cancer

Jamie K. Miller,<sup>1</sup> David L. Shattuck,<sup>1</sup> Ellen Q. Ingalla,<sup>1</sup> Lily Yen,<sup>1</sup> Alexander D. Borowsky,<sup>2</sup> Lawrence J.T. Young,<sup>2</sup> Robert D. Cardiff,<sup>2</sup> Kermit L. Carraway III,<sup>1</sup> and Colleen Sweeney<sup>1</sup>

<sup>1</sup>University of California Davis Cancer Center and <sup>2</sup>Department of Medical Pathology and Laboratory Medicine, School of Medicine, University of California at Davis, Sacramento, California

## Abstract

**The ErbB2 receptor tyrosine kinase is overexpressed in ~25% of breast tumors and contributes to poor patient prognosis and therapeutic resistance. Here, we examine the role of the recently discovered ErbB negative regulator LRIG1 in ErbB2<sup>+</sup> breast cancer. We observe that LRIG1 protein levels are significantly suppressed in ErbB2-induced mammary tumors in transgenic mice as well as in the majority of ErbB2<sup>+</sup> human breast tumors. These observations raise the possibility that LRIG1 loss could contribute to the initiation or growth of ErbB2<sup>+</sup> breast tumors. RNA interference-mediated knock-down of endogenous LRIG1 in the ErbB2-overexpressing breast tumor cell lines MDA-MB-453 and BT474 further elevates ErbB2 in these cells and augments cellular proliferation. In contrast, ectopic expression of LRIG1 reverses these trends. Interestingly, we observe that LRIG1 protein levels are suppressed in response to ErbB receptor activation in breast tumor cells but are unaffected by ErbB activation in immortalized nontransformed breast epithelial cells. Our observations indicate that the suppression of LRIG1 protein levels is a common feature of breast tumors. Moreover, our observations point to the existence of a feed-forward regulatory loop in breast tumor cells where aberrant ErbB2 signaling suppresses LRIG1 protein levels, which in turn contributes to ErbB2 overexpression.** [Cancer Res 2008;68(20):8286–94]

## Introduction

Receptor tyrosine kinases play essential roles in tissue development and homeostasis and their functional inactivation has dramatic consequences on the viability of the organism. In healthy tissue, receptor activation or “positive signaling” is tightly controlled by ligand availability and is counterbalanced by a network of negative regulatory molecules that serve to limit signal output. Disruption of the precise balance between “positive” and “negative” signals is implicated in the development of several diseases, including cancer (1). Ligand-induced down-regulation is the most widely studied attenuation mechanism for receptor tyrosine kinases and uncoupling receptors from this pathway leads to aberrant signaling and cellular transformation (2).

The ErbB family of receptor tyrosine kinases, which includes epidermal growth factor receptor (EGFR), ErbB2/Her2/Neu, ErbB3, and ErbB4, forms a complex signaling network driven by ligand-

stimulated receptor heterodimerization. ErbB2 is overexpressed in 20% to 30% of human breast cancers and correlates with poor patient prognosis and therapeutic resistance (3). The ErbB2/ErbB3 heterodimer functions as an “oncogenic unit” and both receptors are essential for breast tumor cell proliferation (4). In support of this, ErbB2 and ErbB3 overexpression frequently overlap in human breast tumors (5). Moreover, tumors from transgenic mice that express activated ErbB2 in the mammary gland also overexpress ErbB3 (6).

ErbB receptors are subject to strenuous regulation and loss or suppression of negative regulatory proteins unleashes potent growth and survival signals. For example, underexpression of the pan-ErbB inhibitor RALT/Mig-6 in ErbB2-amplified breast cancer cells heightens tumor cell proliferation and favors Herceptin resistance (7). Similarly, loss of the ErbB3 negative regulator Nrdp1 in breast cancer enhances ErbB2/ErbB3-driven tumor cell proliferation and motility (8). Aberrant activation of Src promotes degradation of the ubiquitin ligase c-Cbl, enhancing EGFR stability and providing a mechanism by which Src and EGFR cooperate to drive tumorigenesis (9). These studies suggest that full oncogenic signaling by receptor tyrosine kinases requires the suppression of negative regulators and that loss of negative regulatory proteins may cooperate with receptor gene amplification to yield a permissive environment for receptor overexpression. Strategies that augment or restore expression of endogenous negative regulatory molecules may prove effective in the clinic.

LRIG1 is a transmembrane leucine-rich repeat protein and a recently identified negative regulator of all members of the ErbB family as well as the Met receptor tyrosine kinase (10–12). LRIG1 functions by enhancing the proteolytic degradation of its targets and was identified based on its homology with Kekk-1, a negative regulator of *Drosophila* EGFR. LRIG1-deficient animals develop a psoriasiform epidermal hyperplasia, consistent with deregulated EGFR signaling and a growth-suppressive role for LRIG1 in the skin (13). Analysis of LRIG1 expression *in vivo* reveals that it is decreased in several different tumor types, including squamous cell and renal cell carcinoma and advanced cervical cancer (14–16). As such, LRIG1 has been proposed to be a tumor suppressor (17). However, LRIG1 expression in certain tumor types has been reported to be heterogeneous; for example, colorectal cancers both underexpress and overexpress LRIG1 (18). A recent study involving a few breast tumors reported that LRIG1 expression was increased in five of nine tumors, suggesting that LRIG1 may not function as a growth suppressor in the breast (19). As this finding is inconsistent with its role in suppressing ErbB2 function, we sought to clarify the role of LRIG1 in breast cancer.

In this study, we find that LRIG1 is suppressed in human breast tumors and that the majority of ErbB2-overexpressing breast tumors underexpress LRIG1. This finding is paralleled in a well-characterized transgenic mouse model of ErbB2<sup>+</sup> breast cancer.

**Requests for reprints:** Colleen Sweeney, University of California Davis Cancer Center, University of California at Davis, Research Building III, Room 1100A, 4645 2nd Avenue, Sacramento, CA 95817. Phone: 916-734-0726; Fax: 916-734-0190; E-mail: casweeney@ucdavis.edu.

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RNA interference (RNAi)-mediated depletion studies show that LRIG1 acts to suppress breast tumor cell growth. Interestingly, activation of ErbB2 in breast cancer cells leads to a significant loss of endogenous LRIG1 expression, showing that ErbB2 oncogenic signaling actively contributes to suppression of LRIG1. These data suggest that ErbB2 contributes to its own overexpression by suppressing negative regulators that oppose its action.

## Materials and Methods

**Reagents and cell culture.** Human breast cell lines BT474, MCF-7, MDA-MB-453, MDA-MB-361, SKBR-3, and T47D cells were purchased from the American Type Culture Collection and cultured in the recommended medium (Mediatech) with 10% FCS (Invitrogen) and penicillin-streptomycin antibiotics (Mediatech). Serum-starved medium contained only 0.1% FCS. HMEC4 and HMEC6 cells were kind gifts from Dr. Krishna Rao (Division of Hematology and Oncology, Department of Internal Medicine, Southern Illinois University School of Medicine, Springfield, IL) and were grown in DFCI-1 medium as described (20). Neuregulin-1 $\beta$  (Nrg1) was produced and purified as previously described (21). The EGFR/ErbB2 inhibitor 4557W was purchased from EMD Biosciences. Antibodies used in these studies include anti-LRIG1-151 (AgriSera); anti-ErbB2 Ab3 (EMD Biosciences); anti-ErbB3 C-17 (Santa Cruz Biotechnology); anti-phosphorylated ErbB2 (p-ErbB2) 6B12, anti-phosphorylated Akt (p-Akt) S473, anti-total Akt, anti-phosphorylated extracellular signal-regulated kinase (p-ERK) 1/2, and anti-total ERK1/2 (Cell Signaling Technology); anti-myc (Invitrogen); anti-phosphotyrosine (BD Transduction); and anti-tubulin and anti-actin AC-15 (Sigma). FuGene6 was purchased from Roche and transfections were performed as recommended by the manufacturer.

**Human breast tissue analysis.** Frozen human tissues from clinical samples were provided by the National Cancer Institute Cooperative Human Tissue Network and the University of California at Davis Cancer Center Specimen Repository. All of the samples were approved for laboratory use by the institutional review board of the University of California at Davis School of Medicine. Samples were homogenized in 10  $\mu$ L T-PER (Pierce) per mg of tissue in the presence of 4  $\mu$ g/mL leupeptin, 4  $\mu$ g/mL pepstatin, 4  $\mu$ g/mL aprotinin, and 100 nmol/L AEBSF and then centrifuged to remove insoluble products.

**Transgenic mice.** NDL mice expressing the constitutively active ErbB2/Neu transgene under the control of the mouse mammary tumor virus promoter (NDL2-5; 6) were bred and maintained at the animal facilities at the University of California at Davis. Following tumor development, mammary fat pad tissue of the tumor and adjacent normal were collected and snap frozen in liquid nitrogen. Tissue lysates were prepared as described for the human tissues. To produce a pure epithelial NDL2-5 tumor line, one mammary tumor  $\sim$  1 cm in diameter was excised from a 7-mo-old FvB NDL2-5 mouse. One 2-mm cross-section was fixed in formalin, paraffin embedded, and stained with H&E for histologic validation. The remainder of the tumor was rinsed twice with PBS, minced with razor blades to form a slurry of cells in 25 mL of lysis buffer [20 mL DMEM/F12, 5 mL bovine serum albumin (7.5% w/v in water), 10  $\mu$ L hydrocortisone, 200  $\mu$ L of 1 mol/L HEPES, 10  $\mu$ L cholera toxin (20  $\mu$ g/mL), 10  $\mu$ L insulin (10 mg/mL), 3 mg/mL collagenase] and then gently agitated overnight at 37°C. Cells were spun at 80  $\times$  g for 1.5 min, washed once with DMEM/F12, and then spun at 200  $\times$  g for 4 min before plating in growth medium (DMEM with 10% fetal bovine serum and penicillin/streptomycin antibiotics). After passaging five times using differential trypsinization, cells were filtered through a 40- $\mu$ m filter to produce a single-cell suspension and labeled with anti-CD49f. Cells were then sorted using fluorescence-activated cell sorting to eliminate fibroblast contamination using a narrow gate for strong CD29f signal, and the resulting epithelial cells were plated in growth medium.

**Real-time PCR analysis.** Total RNA was harvested from cells using Trizol followed by the Micro-to-Midi Total RNA Purification System (Invitrogen). The High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) was used to convert 5  $\mu$ g RNA into cDNA. Real-time analysis was carried out with a Bio-Rad iCycler iQ Real Time PCR instrument using

Applied Biosystems Taqman Gene Expression primers and probes that were labeled with FAM and EuroGentec Two Step RT qPCR Master Mix.

**Cell signaling and growth following transduction.** Breast cancer cell lines were virally transduced as described previously (12), and MDA-MB-453 and SKBR-3 cells were selected with 0.75 and 1.75  $\mu$ g/mL of puromycin, respectively. To analyze receptor phosphorylation and downstream signaling, serum-starved cells in 12-well plates (Nunc) were treated with Nrg1 for various times at 37°C. Lysates were collected in sample buffer (62.5 mmol/L Tris-HCl, 2% SDS, 5%  $\beta$ -mercaptoethanol, 10% glycerol, 0.05% bromophenol blue), boiled for 5 min at 95°C, and resolved by 8% SDS-PAGE. Following transfer to nitrocellulose (Pall Life Sciences), blots were cut horizontally into strips and immunoblotted with various primary antibodies. Blotted proteins were detected using horseradish peroxidase-conjugated secondary antibodies (Invitrogen) and developed using the SuperSignal West chemicals (Pierce). An Alpha Innotech imaging station with AlphaEase FC software was used to capture and quantify images. All results are representative of at least three independent experiments.

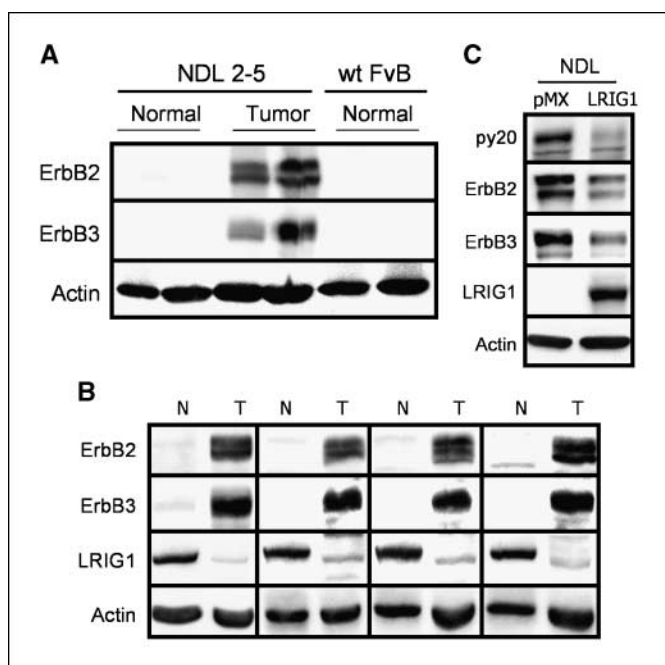
To measure proliferation, MDA-MB-453 and SKBR3 cells were plated into 24-well plates (Corning) at a density of  $1 \times 10^5$  and  $2.5 \times 10^4$  per well, respectively. After 24 h, cells were placed in conditioned medium and allowed to proliferate for 48 h. During the last 2 h of growth, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the medium to measure activity. Crystals formed from the MTT were dissolved in acidic isopropanol and the absorption was measured at 570 nm with a baseline subtraction at 655 nm.

**Cell assays using RNAi.** MDA-MB-453 and BT474 cells were plated at a density of  $1 \times 10^5$  and  $2.5 \times 10^4$  per well, respectively, in 24-well plates (Corning). After settling for 16 h, cells were transfected with 100 nmol/L Dharmacon nontargeting or LRIG1 ON-TARGETplus SMARTpool RNAi per manufacturer's directions (Dharmacon). Proliferation was measured after 48 h as described above. For cell signaling experiments, 48 h after transfection, cells were serum starved overnight. Various Nrg1 concentrations in serum-starved medium were added to the cells for 15 min followed by cell lysis in sample buffer. Lysates were then analyzed via Western blot as described.

**Statistical analysis.** Values are expressed as mean  $\pm$  SE unless noted. *P* values were determined using the two-tailed Student's *t* test with values  $<0.05$  considered statistically significant (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ).

## Results

**LRIG1 is suppressed in ErbB2-overexpressing mouse mammary tumors.** Transgenic overexpression of activated ErbB2 (NDL2-5 or Neu deletion mutant) in the mouse mammary epithelium yields focal adenocarcinomas that evolve after a long latency and metastasize to the lung with high frequency (22). Previous studies have shown that endogenous ErbB3 protein levels are elevated 10- to 15-fold in NDL tumor tissue, underscoring the link between ErbB2 and ErbB3 in mammary tumorigenesis (6). Interestingly, this increase in ErbB3 protein expression was accompanied by a rather modest ( $<3$ -fold) elevation in ErbB3 transcript levels, suggesting that posttranscriptional mechanisms such as enhanced message translation and/or augmented receptor stability contribute to ErbB3 overexpression in NDL tumors. These mechanisms may also be involved in human breast cancer, as ErbB3 gene amplification has not been detected despite ErbB3 protein overexpression. To further explore this, we examined the expression of ErbB2 and ErbB3 in normal mammary gland from nontransgenic FvB littermates, in normal mammary gland from NDL2-5 transgenic mice, and in tumor tissue from NDL2-5 transgenic mice by Western blotting (Fig. 1A). Endogenous ErbB3 protein expression was significantly elevated in tumor tissue, as previously reported (6). Interestingly, a similar result was observed with ErbB2. ErbB2 was very modestly expressed in nontransgenic normal mammary gland and in normal mammary gland from



**Figure 1.** LRIG1 is suppressed in tumors from MMTV-ErbB2 mice. *A*, normal mammary tissue from nontransgenic FvB littermates was collected along with normal and tumor tissue from MMTV-NDL2-5 mice. Lysates from these tissues were blotted for ErbB2, ErbB3, and actin as a loading control. *B*, tumor (T) and adjacent normal (N) tissue were collected from the mammary fat pad of NDL2-5 transgenic mice. Lysates from these tissues were blotted for ErbB2, ErbB3, LRIG1, and actin. *C*, an epithelial cell line produced from an NDL2-5 tumor was transfected with myc-tagged LRIG1 or control vector pMX. Lysates were immunoblotted with antibodies to phosphotyrosine (*py20*), ErbB2, ErbB3, LRIG1, and actin.

transgenic animals but was abundantly expressed in tumor tissue. Because the transgene is present in both normal and tumor tissue in transgenic animals, these results suggest that ErbB2 gene amplification is not sufficient for ErbB2 protein overexpression. Rather, it suggests that events that occur specifically in the tumor tissue, among them the suppression of negative regulatory molecules, may cooperate with gene amplification to yield maximal ErbB2 protein overexpression.

We next examined the expression of LRIG1 in adjacent normal and tumor tissue from the NDL2-5 mice. Tissue from 10 independent mice was examined by Western blotting; four representative samples are shown in Fig. 1*B*. Both ErbB2 and ErbB3 protein expression were dramatically increased in tumor tissue and this could not be explained by a significant difference in receptor transcript levels as measured by real-time PCR analysis. (In each case, transcript levels in normal tissue were normalized to 1.00. Values are an average of four mice: Neu,  $0.92 \pm 0.19$ ; mErbB3,  $1.17 \pm 0.30$ .) Remarkably, all animals examined showed a dramatic loss of LRIG1 expression in tumor tissue. These results raise the possibility that LRIG1 suppression may contribute to ErbB2 overexpression in breast tumors. Real-time PCR analysis of LRIG1 transcript levels in normal and tumor tissue revealed a 40% decrease in tumor tissue. (Transcript levels in normal tissue were normalized to 1.00. Values are an average of four mice: mLRIG1,  $0.58 \pm 0.09$ ).

To further examine the role of LRIG1 loss in ErbB2/ErbB3-overexpressing NDL2-5 tumors, an NDL2-5 cell line was established from a primary tumor. This cell line maintained robust expression

of ErbB2 and ErbB3 and lacked detectable endogenous LRIG1 by Western blotting (Fig. 1*C*). To examine whether ectopic expression of LRIG1 was sufficient to diminish ErbB2/ErbB3 expression, the NDL2-5 cell line was stably transduced with either control virus (pMX) or virus expressing myc-tagged LRIG1. As shown in Fig. 1*C*, expression of LRIG1 resulted in a dramatic loss of both receptors. These results indicate that the loss of LRIG1 observed in NDL2-5 tumors is functionally relevant.

**LRIG1 expression is decreased in human breast cancer.** To determine whether the loss of LRIG1 observed in murine mammary tumors was reflected in human breast tumors, LRIG1 expression was examined by Western blotting of a panel of normal ( $n = 42$ ) and tumor ( $n = 67$ ) tissues. In a comparison of all normal to all tumor, over three fifths of the tumors (42 of 67) showed a loss of LRIG1 protein (Fig. 2*A*), and on average, LRIG1 protein levels were decreased by 33% in tumors compared with normal tissues (*inset*;  $P < 0.02$ ).

Richardson-Bloom tumor grade information was available for 58 of the 67 tumors in our collection, enabling us to examine whether LRIG1 protein expression varied with tumor grade. Because grade 1 samples were rare in our cohort, tumors were sorted into two classes: low-grade tumors, consisting of grades 1 and 2 ( $n = 26$ ), and high-grade (grade 3) tumors ( $n = 32$ ). Figure 2*B* shows a plot of relative LRIG1 expression in each of these classes. Strikingly, there is on average a 2-fold decrease in LRIG1 levels in the high-grade tumors compared with those of low grade (*inset*;  $P < 0.005$ ).

Because a decrease in LRIG1 transcript was observed in tumors from NDL2-5 mice, we were interested in determining whether LRIG1 transcript was also decreased in human breast cancer. We queried the Oncomine database,<sup>3</sup> a collection of microarray data from more than 18,000 cancer gene expression profiles (23). Analysis of LRIG1 transcript in the breast revealed a statistically significant underexpression of LRIG1 transcript in tumors compared with normal tissue (Fig. 2*C, left*; ref. 24) and an inverse correlation with tumor grade (Fig. 2*C, right*; refs. 25, 26). Collectively, these observations indicate that LRIG1 protein and transcript are decreased in both murine and human breast tumors relative to normal tissue, suggesting that loss of LRIG1 may be beneficial to tumor growth or survival.

**LRIG1 expression is decreased in ErbB2<sup>+</sup> human breast cancer.** Of the 67 tumor specimens in our cohort, 13 were ErbB2<sup>+</sup> tumors (19.4%) as indicated by their pathology reports. A significant decrease in LRIG1 expression was observed in ErbB2-overexpressing murine mammary tumors (Fig. 1*B*). To determine whether LRIG1 expression is also decreased in ErbB2<sup>+</sup> human breast cancer, the level of LRIG1 in each tumor was examined by Western blotting and directly compared with its patient-matched normal tissue control. As shown in Fig. 3*A*, decreased LRIG1 expression was observed in 76% (10 of 13) of ErbB2<sup>+</sup> breast tumors compared with the matched normal tissue, whereas 2 tumors exhibited an increase in LRIG1 expression. To further support these findings, an Oncomine query revealed a loss of LRIG1 transcript in ErbB2<sup>+</sup> tumors ( $n = 33$ ) compared with ErbB2<sup>-</sup> tumors ( $n = 99$ ; ref. 27), as shown in Fig. 3*B* ( $P = 0.039$ ). These findings indicate that LRIG1 protein expression and transcript levels are decreased in the majority of ErbB2<sup>+</sup> human breast cancers as well as in mammary tumors from ErbB2-overexpressing mice. The consistency in this

<sup>3</sup> <http://www.oncomine.org>

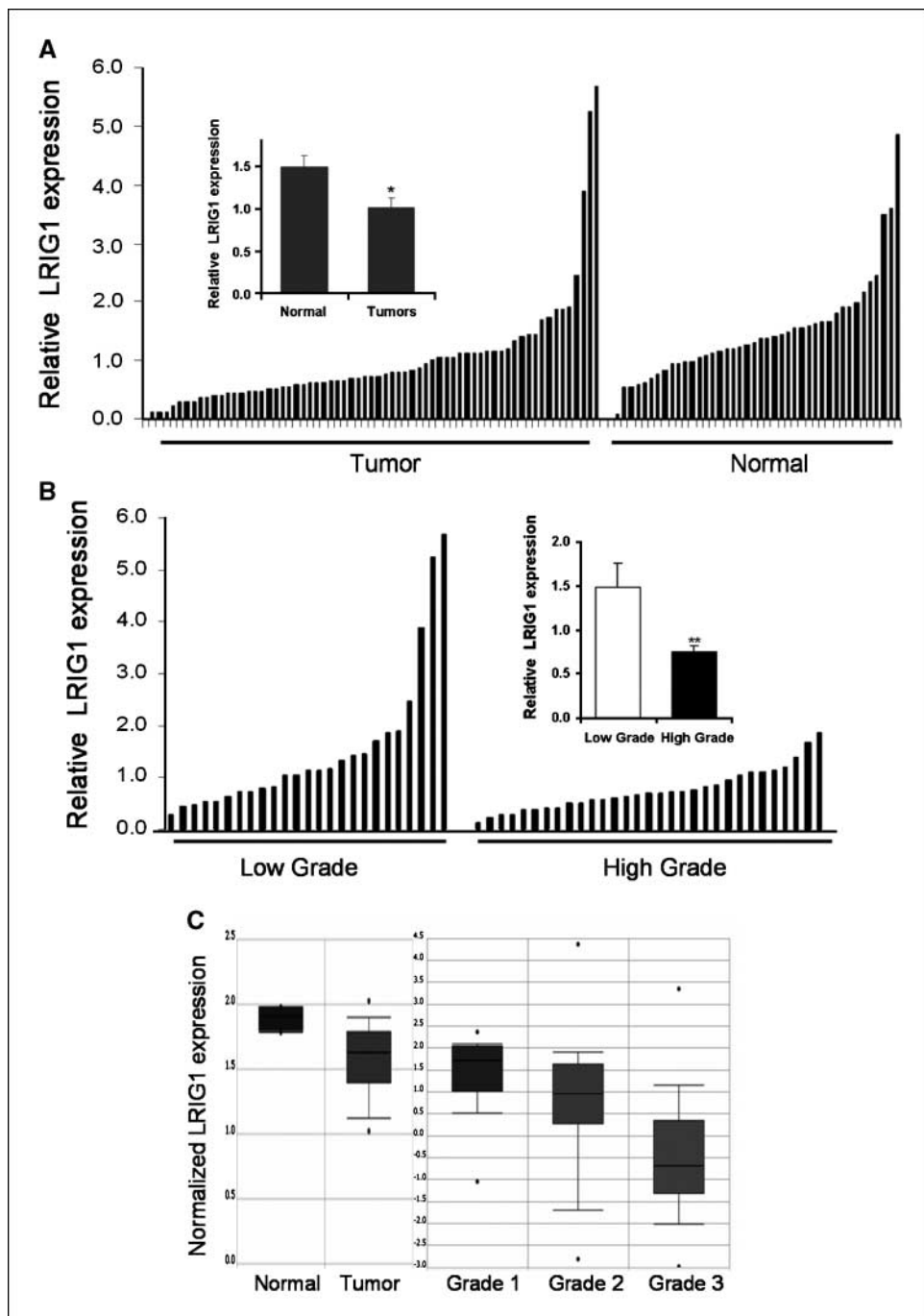
result suggests that LRIG1 loss may provide ErbB2-overexpressing tumors with a selective advantage.

**Depletion of LRIG1 in ErbB2-overexpressing breast cancer cells enhances tumor cell growth.** To model the loss of LRIG1 observed in ErbB2<sup>+</sup> breast cancer, LRIG1 was depleted in breast tumor cells by RNAi-mediated silencing and the effect on ErbB2 expression was examined. A survey of several ErbB2-overexpressing breast tumor cells found that MDA-MB-453 and BT474 cells express detectable levels of LRIG1 protein (Fig. 4A). As shown in Fig. 4A, LRIG1-specific RNAi efficiently reduced LRIG1 protein levels in both cell lines compared with the nontargeting control. This reduction in LRIG1 brought about a >30% increase in ErbB2 expression ( $P = 0.002$ , MDA-MB-453;  $P = 0.007$ , BT474) as well as an

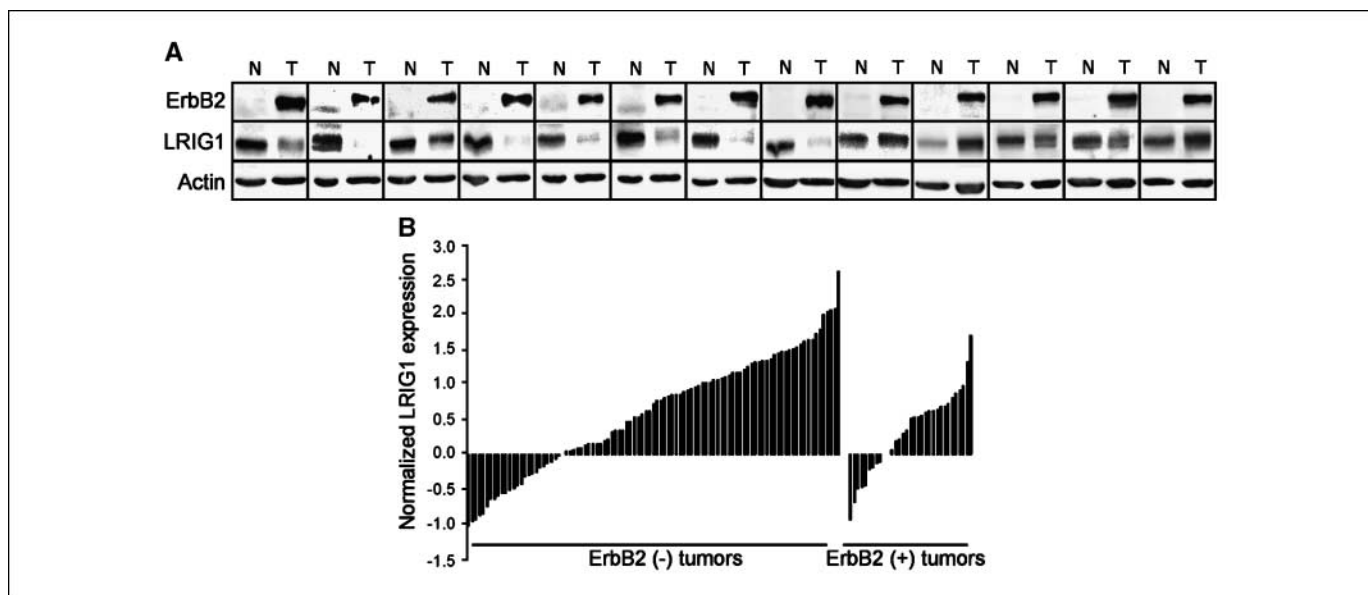
increase in basal ErbB2 phosphorylation in both cell lines. These findings show that depletion of LRIG1 is sufficient to further increase ErbB2 expression in ErbB2-overexpressing breast cancer cells.

We next examined the effect of LRIG1 loss on growth factor-stimulated signal transduction. As shown in Fig. 4B, depletion of LRIG1 in MDA-MB-453 cells augmented basal Akt phosphorylation and sensitized cells to Nrg1. Mitogen-activated protein kinase (MAPK) activation was not similarly affected. Accumulation of ErbB2/ErbB3 at the plasma membrane of breast cancer cells has previously been shown to selectively heighten signaling through the phosphatidylinositol 3-kinase/Akt pathway (21). Because depletion of LRIG1 causes accumulation of

**Figure 2.** LRIG1 levels are decreased in human breast tumors. *A*, relative LRIG1 expression was compared in breast tumor and normal specimens via Western blot analysis. Each of the 67 tumor and 42 normal samples is plotted in order of increasing LRIG1 levels after normalizing to actin levels. *Inset, columns*, mean value of LRIG1 protein expression for each subset; *bars*, SE. *B*, using the Richardson-Bloom grade information available from the pathology reports of 58 of the 67 tumors, relative LRIG1 expression of each tumor is plotted as a function of the tumor grade. Low grade represents tumor grades of 1 and 2, with high grade corresponding to grade 3 tumors. *Right, inset, columns*, average LRIG1 level of each grouping; *bars*, SE. *C*, representative plots of normalized LRIG1 transcript in breast tissue from the Oncomine database are pictured. Each study has a  $P$  value of <0.05.



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**Figure 3.** ErbB2<sup>+</sup> human tumors display reduced LRIG1 expression. *A*, lysates of ErbB2<sup>+</sup> tumors and patient-matched normal samples were immunoblotted with antibodies to ErbB2, LRIG1, and actin. *B*, the Hess study available through OncoPrint (27) was queried for transcript expression of LRIG1 in breast tumors and is plotted based on ErbB2 status of the tumors.

ErbB receptors at the cell surface (28, 29), this provides a likely mechanism by which Akt signaling is heightened.

To determine whether LRIG1 depletion affected breast tumor cell growth, control- and LRIG1-targeted cells were subjected to a MTT proliferation assay. As shown in Fig. 4C, depletion of LRIG1 augmented basal (ligand independent) growth in both cell lines. To determine whether the increase in basal growth was due to an increase in constitutive ErbB2 signaling, basal growth of MDA-MB-453 cells was examined in the presence of 4557W, a small-molecule inhibitor of EGFR and ErbB2. 4557W efficiently reduced ErbB2 phosphorylation (Fig. 4D, top). Depletion of LRIG1 augmented basal cell growth and this advantage was neutralized by 4557W (Fig. 4D, bottom). Because MDA-MB-453 cells do not express detectable amounts of EGFR, these results indicate that LRIG1 loss enhances basal tumor cell growth primarily through an increase in constitutive ErbB2 signaling.

**Ectopic expression of LRIG1 in ErbB2-overexpressing breast cancer cells suppresses tumor cell growth.** We next sought to determine whether ectopic expression of LRIG1 in ErbB2-overexpressing breast cancer cells was sufficient to affect tumor cell growth. As shown in Fig. 5A, MDA-MB-453 and SKBR-3 cells were transduced with a myc-tagged version of LRIG1 and puromycin-resistant clones were pooled to negate the effects of clonal variation. SKBR-3 cells express very limited amounts of endogenous LRIG1 but have abundant ErbB2 (see Fig. 6A). Importantly, ectopic expression of LRIG1 in both cell lines diminished ErbB2 protein expression (Fig. 5A) and decreased basal and Nrg1-stimulated tumor cell proliferation (Fig. 5B). Previous studies from our lab and others have shown that LRIG1 decreases ErbB receptor stability (10, 11). However, LRIG1 has also been reported to decrease EGFR protein and transcript levels when ectopically expressed in neuroglioma cells (30). To determine whether ErbB2 transcript was similarly affected in our model systems, we performed real-time PCR analysis. pMX was normalized to 1.00 in both cases. Interestingly, ErbB2 transcript levels were suppressed in both lines (Fig. 5A). These results suggest that in addition to

destabilizing ErbB2 protein, LRIG1 may suppress ErbB2 transcription or affect ErbB2 transcript stability, perhaps by targeting factors that regulate these processes.

To examine the mechanism by which LRIG1 suppresses tumor cell growth, downstream signaling was examined in LRIG1-transduced MDA-MB-453 cells (Fig. 5C). Ectopic expression of LRIG1 decreased ErbB2 expression and phosphorylation and diminished Nrg1-stimulated Akt phosphorylation but did not have a significant effect on MAPK phosphorylation. These results indicate that the LRIG1 status of breast cancer cells has a significant effect on tumor cell signaling and proliferation.

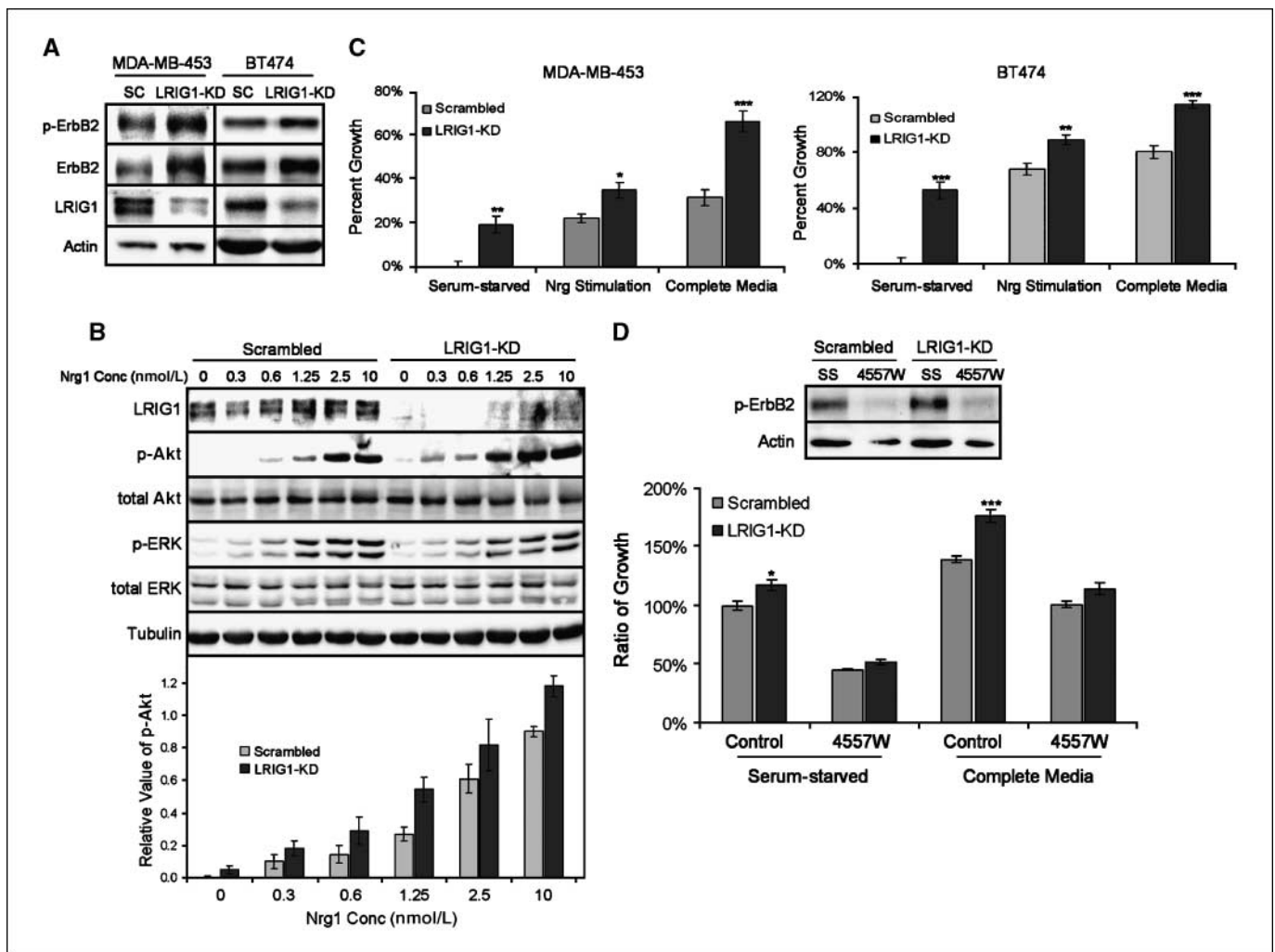
**ErbB2 activation suppresses endogenous LRIG1 expression.** Because LRIG1 expression is decreased in ErbB2-overexpressing murine and human breast tumors, we were interested in examining whether ErbB2 activation may contribute to suppression of LRIG1, in effect promoting its own stability. We first examined endogenous LRIG1 expression in a panel of ErbB2-overexpressing breast cancer cell lines with different levels of constitutive ErbB2 activation as shown in Fig. 6A. Interestingly, the two cell lines with the highest levels of phospho-ErbB2 expressed the lowest amount of endogenous LRIG1. To examine whether activation of ErbB2 is sufficient to suppress LRIG1 expression, we used MCF-7 breast tumor cells, which express modest amounts of endogenous ErbB2. MCF-7 cells were transiently transfected with a constitutively active form of ErbB2, NeuT, and endogenous LRIG1 levels were examined by Western blotting as shown in Fig. 6B (left). Expression of NeuT was sufficient to decrease endogenous LRIG1 protein levels by 21% ( $21 \pm 4$ ;  $P = 0.0001$ ;  $n = 8$  independent experiments) and this was accompanied by a 15% decrease in LRIG1 transcript levels as determined by real-time PCR analysis. This is likely an underestimation of the effect of chronic ErbB2 activation on endogenous LRIG1 expression, as these cells were transiently transfected and were not a pure population of NeuT-expressing cells. To further implicate ErbB2 in negative regulation of LRIG1 expression, endogenous ErbB2 was silenced by small interfering RNA in MCF-7 cells and LRIG1 protein expression was examined by

Western blotting (Fig. 6B, right). Following ErbB2 knockdown, there was a significant increase in endogenous LRIG1 expression, underscoring the reciprocal relationship between ErbB2 and LRIG1.

We next examined whether activation of endogenous ErbB2 by ligand stimulation was sufficient to affect endogenous LRIG1 expression. Although this means of ErbB2 activation is transient, we were interested in determining its effect on LRIG1 expression. For this experiment, we chose tumor cells that expressed moderate amounts of LRIG1 so that any changes in LRIG1 expression could be easily quantified. MCF-7 and T47D cells were stimulated with Nrg1, and 24 h later, LRIG1 expression was examined by Western blotting as shown in Fig. 6C. Interestingly, ligand stimulation of both cell lines led to a significant decline in LRIG1 protein expression (Fig. 6C). LRIG1 transcript levels also decreased following ligand stimulation, although the magnitude of this

decrease varied between the two cell lines (MCF-7,  $0.89 \pm 0.05$ ; T47D,  $0.26 \pm 0.05$ ). These results show that activation of ErbB2 in breast tumor cells leads to the suppression of the negative regulator LRIG1, at least in part through a decrease in transcript levels. Because LRIG1 depletion by RNAi enhances ErbB2 expression (Fig. 4A), these results uncover a mechanism by which ErbB2 may contribute to or sustain its own overexpression.

To determine whether suppression of LRIG1 was specific to transformed cells, LRIG1 expression was examined in two different human mammary epithelial cell lines, HMEC4 and HMEC6 (20), as shown in Fig. 6C. Western blotting of these cell lines revealed modest but detectable expression of ErbB2, which could be activated by treatment of cells with Nrg1 (data not shown). Interestingly, stimulation of these cells with Nrg1 for 24 h did not result in a decrease in LRIG1 expression (quantified in Fig. 6D). A



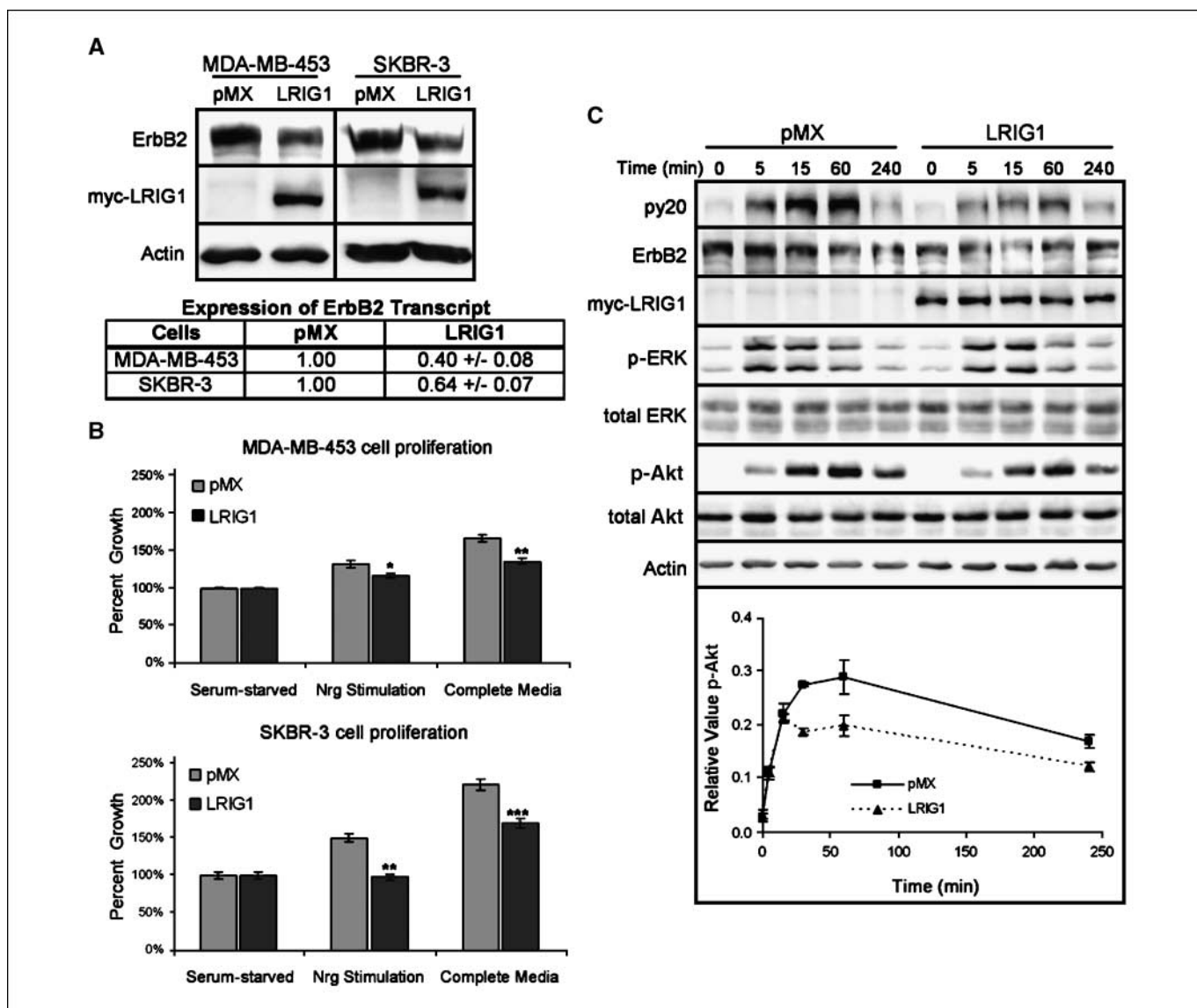
**Figure 4.** Depletion of LRIG1 augments ErbB2 expression and enhances cellular proliferation. *A*, lysates from MDA-MB-453 and BT474 cells transfected for 72 h with RNAi oligos targeting LRIG1 (*LRIG1-KD*) or scrambled control (*SC*) were blotted with antibodies to p-ErbB2, ErbB2, LRIG1, and actin. *B*, 48 h after transfecting with RNAi oligos, MDA-MB-453 cells were serum starved overnight and then treated with increasing concentrations of Nrg1 for 15 min. Whole-cell lysates were blotted with antibodies to LRIG1, p-Akt, total Akt, p-ERK, total ERK, and tubulin as a loading control. *Bottom*, densitometric analysis of the average p-Akt signal from three independent experiments for each Nrg1 concentration along with SE. *C*, 24 h after transfecting cells with RNAi oligos as described above, MDA-MB-453 and BT474 cells were placed in serum-starved medium, in the presence or absence of 2.5 nmol/L Nrg1, or complete medium (10% FCS). After 48 h, proliferation was measured by a MTT assay. Three independent experiments were conducted with a representative experiment shown. *Columns*, mean of four replicates within this experiment; *bars*, SE. *D*, 24 h after transfecting cells with RNAi oligos as described above, MDA-MB-453 cells were placed in serum-starved (*SS*) or complete medium in the presence or absence of the EGFR/ErbB2 inhibitor 4557W. After 48 h, proliferation was measured by a MTT assay, with basal proliferation being normalized as 100%. Additionally, whole-cell lysates from serum-starved cells were collected following treatment and blotted for p-ErbB2 and actin (*top*) to verify inhibition of ErbB2 by 4557W.

decrease in LRIG1 expression was not observed at any time following Nrg1 treatment of HMEC4/HMEC6 cells (data not shown). These results indicate that activation of ErbB receptors in breast tumor cells leads to the suppression of endogenous LRIG1 expression, whereas LRIG1 expression is maintained following ErbB activation in normal mammary epithelial cells.

## Discussion

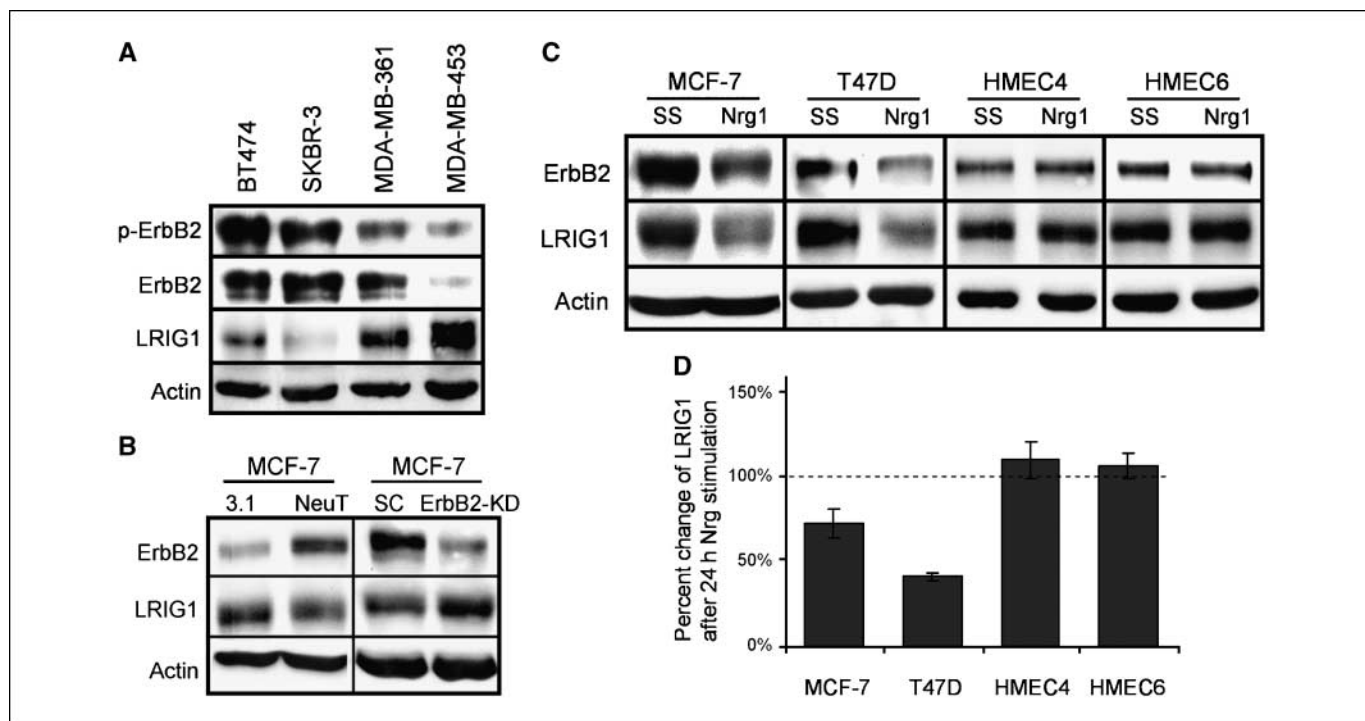
ErbB2 gene amplification in breast cancer and its association with poor patient prognosis was first described by Slamon and colleagues in 1987 (31). Although gene amplification is typically associated with protein overexpression, this correlation is not

absolute. 1<sup>+</sup> protein staining (negative or weak) is observed in the presence of high-level amplification of the *ErbB2* gene (32), suggesting that there are potent mechanisms in place that oppose inappropriate ErbB2 protein expression. Conversely, there is significant evidence of 2<sup>+</sup> (moderate) and 3<sup>+</sup> (high) protein staining in the absence of gene amplification, indicating that gene amplification is not necessary for ErbB2 protein overexpression (33, 34). Our findings with the ND2-5 transgenic mice show that gene amplification is not sufficient for ErbB2 protein overexpression in this model. Rather, the loss or compromise of specific negative regulatory molecules such as LRIG1 in breast tumor cells seems to cooperate with increased gene dosage to yield the most robust ErbB2 protein expression. In agreement with this,



**Figure 5.** Ectopic expression of LRIG1 inhibits breast cancer cell proliferation. **A**, MDA-MB-453 and SKBR-3 cells were transduced with myc-tagged LRIG1 retrovirus or pMX control. Whole-cell lysates were immunoblotted with antibodies for ErbB2, myc, and actin. ErbB2 transcript was measured by real-time PCR analysis. pMX-transduced cells were normalized to 1.0. **B**, MDA-MB-453 and SKBR-3 cells transduced with LRIG1 or control pMX vector were treated with serum-starved medium in the presence or absence of 2.5 nmol/L Nrg1, or with complete medium, for 48 h. Proliferation was then measured via a MTT assay. Each experiment was repeated in triplicate with a representative experiment shown. Columns, mean of four replicates; bars, SE. **C**, MDA-MB-453 cells transduced with LRIG1 or control pMX vector were stimulated with 1.25 nmol/L Nrg1 for various time points. Whole-cell lysates were then immunoblotted with antibodies to phosphotyrosine (py20), ErbB2, myc, p-Akt, total Akt, p-ERK, total ERK, and actin. Densitometric analysis of the p-Akt signal normalized to total Akt is plotted for each time point along with SE from three independent experiments.





**Figure 6.** ErbB2 activation reduces LRIG1 expression in breast tumor cells. *A*, whole-cell lysates collected from BT474, SKBR-3, MDA-MB-361, and MDA-MB-453 cells were blotted with antibodies to p-ErbB2, ErbB2, LRIG1, and actin. *B*, MCF-7 cells were transiently transfected for 48 h with NeuT or control vector pcDNA3.1 (3.1) and cell lysates were blotted for ErbB2, LRIG1, and actin. Additionally, MCF-7 cells were treated with 100 nmol/L scrambled control (SC) or ErbB2 RNAi oligos (*ErbB2-KD*) for 48 h. Lysates were collected and blotted for ErbB2, LRIG1, and actin. *C*, MCF-7, T47D, HMEC4, and HMEC6 cells were treated with serum-starved medium in the presence or absence of 10 nmol/L Nrg1. After 24 h, whole-cell lysates were collected and samples were immunoblotted with antibodies to ErbB2, LRIG1, and actin. *D*, densitometric analysis of LRIG1 protein expression from each cell line in *C* is plotted along with SE from at least four independent experiments.

LRIG1 is poorly expressed in ErbB2<sup>+</sup> human breast tumors and RNAi-mediated depletion of LRIG1 in ErbB2-amplified breast cancer cells further enhances ErbB2 expression and tumor cell growth.

Recent studies have begun to provide some insight into endogenous mechanisms that limit ErbB2 expression. Foxp3, a member of the forkhead/winged helix transcription factor family, is an X-linked transcriptional repressor of ErbB2 and loss or mutational inactivation of Foxp3 is strongly correlated with ErbB2 overexpression in breast cancer. Importantly, in ErbB2-amplified breast cancers, Foxp3<sup>+</sup> tumors express less ErbB2 protein compared with Foxp3<sup>-</sup> tumors, indicating that Foxp3 resists ErbB2 overexpression even in the amplified setting (35). Loss of the 14-3-3 $\sigma$  tumor suppressor may provide a distinct mechanism for ErbB2 overexpression. The EGR2/Krox20 transcription factor and its coactivator CITED1 are up-regulated in the MMTV-Cre/Flox-Neo-NeuNT mammary tumor model, a model that uses activated ErbB2 under the control of its endogenous promoter (36). Interestingly, 14-3-3 $\sigma$  sequesters EGR2 in the cytoplasm and ectopic expression of 14-3-3 $\sigma$  decreases ErbB2 expression in mammary tumor cells.

Posttranscriptional regulators of ErbB2 include LRIG1, RALT/Mig-6, and the chaperone-dependent ubiquitin ligase CHIP (37). The expression of CHIP in breast cancer has not yet been examined. RALT/Mig-6, a feedback inhibitor of the ErbB family, suppresses receptor catalytic activity through interaction with the kinase domain. ErbB2-overexpressing breast cancer cells are defective in their transcriptional induction of RALT/Mig-6 (7) and the *RALT/Mig-6* gene is down-regulated in breast cancer

patients with shortened survival (38). Such studies illustrate the diversity of mechanisms used by cells in their strict regulation of ErbB2 signaling and the consequences of the loss of these endogenous regulators.

In this study, we provide evidence that LRIG1 is a growth suppressor in breast cancer. We show that LRIG1 expression is decreased in human breast cancer and that the majority of ErbB2<sup>+</sup> tumors underexpress LRIG1. This is reflected in a transgenic mouse model of ErbB2<sup>+</sup> breast cancer. Depletion of residual LRIG1 in ErbB2-overexpressing breast cancer cells further augments ErbB2 expression and tumor cell proliferation, whereas ectopic expression of LRIG1 decreases ErbB2 expression and attenuates tumor cell proliferation. We show that activation of ErbB2 in breast tumor cells is sufficient to decrease LRIG1 expression, at least in part through decreases in LRIG1 transcript, suggesting that ErbB2 takes an active role in its own overexpression through the marginalization of negative regulatory pathways. Suppression of negative regulatory pathways would create an environment in which inappropriate expression of ErbB2 would not be effectively policed. Depending on timing and cellular context, this could contribute to tumor initiation, progression, or metastasis.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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