

β -Catenin Signaling Is a Critical Event in ErbB2-Mediated Mammary Tumor Progression

Babette Schade¹, Robert Lesurf^{1,2}, Virginie Sanguin-Gendreau¹, Tung Bui¹, Geneviève Deblois¹, Sandra A. O'Toole^{3,5,8,9}, Ewan K.A. Millar^{3,4,5,6}, Sara J. Zardawi³, Elena Lopez-Knowles^{10,11}, Robert L. Sutherland^{5,9}, Vincent Giguère¹, Michael Kahn, Michael Hallett^{1,2}, and William J. Muller¹

Abstract

Although ERBB2 amplification and overexpression is correlated with poor outcome in breast cancer, the molecular mechanisms underlying the aggressive nature of these tumors has not been fully elucidated. To investigate this further, we have used a transgenic mouse model of ErbB2-driven tumor progression (ErbB2^{K1} model) that recapitulates clinically relevant events, including selective amplification of the core *erbB2* amplicon. By comparing the transcriptional profiles of ErbB2^{K1} mammary tumors and human ERBB2-positive breast cancers, we show that ErbB2^{K1} tumors possess molecular features of the basal subtype of ERBB2-positive human breast cancer, including activation of canonical β -catenin signaling. Inhibition of β -catenin-dependent signaling in ErbB2^{K1}-derived tumor cells using RNA interference impaired tumor initiation and metastasis. Furthermore, treatment of ErbB2^{K1} or human ERBB2-overexpressing tumor cells with a selective β -catenin/CBP inhibitor significantly decreased proliferation and ErbB2 expression. Collectively, our data indicate that ERBB2-mediated breast cancer progression requires β -catenin signaling and can be therapeutically targeted by selective β -catenin/CBP inhibitors. *Cancer Res*; 73(14); 4474–87. ©2013 AACR.

Introduction

The progression of normal mammary epithelial cells to a malignant phenotype involves multiple genetic events including amplification and overexpression of proto-oncogenes, such as *ERBB2* (Neu, HER2; ref. 1). ERBB2 amplification and subsequent overexpression strongly correlates with a negative clinical prognosis in both lymph node-positive and -negative breast cancer (2). Direct evidence supporting a role for ERBB2 in human breast cancer derives from observations made with transgenic mice. Overexpression of activated ErbB2 in the

mammary epithelium results in the rapid induction of metastatic multifocal mammary tumors (reviewed in ref. 3). One limitation of these studies is that ErbB2 expression is driven by the hormonally regulated mouse mammary tumor virus (MMTV) promoter, resulting in nonphysiologic expression of the transgene. In an attempt to more closely mimic the events involved in ErbB2-induced mammary tumor progression, we have generated transgenic mice that carry a Cre-inducible-activated *erbB2* allele under the transcriptional control of the endogenous *erbB2* promoter (herein referred to as the ErbB2^{K1} model; ref. 4). In contrast to the rapid tumor progression observed in the MMTV-regulated ErbB2 strains, focal mammary tumors arose only after an extended latency period in the ErbB2^{K1} model, and this was further associated with a dramatic elevation of both ErbB2 protein and transcript. Remarkably, the elevated expression of ErbB2 was correlated with the selective genomic amplification of the activated *erbB2* allele. Thus, as in human breast cancers, amplification of *erbB2* seems to be a critical event in mammary tumor progression in this unique transgenic mouse model (reviewed in ref. 3).

Another critical event in the development of human cancer is the dysregulation or mutational activation of the Wnt/ β -catenin signaling axis (5). β -Catenin is a multifunctional protein that plays a vital structural role in cadherin-based cell adhesion, and is an important transcriptional activator of canonical Wnt-mediated gene expression (6, 7). Activation of the Wnt/ β -catenin pathway triggers a series of events that inactivates a complex consisting of adenomatous polyposis coli (APC), axin, and glycogen synthase kinase 3 β (GSK3 β). This complex is responsible for β -catenin degradation, and its inactivation promotes accumulation of β -catenin in the

Authors' Affiliations: ¹Goodman Cancer Centre; ²McGill Centre for Bioinformatics, McGill University, Montreal, Quebec, Canada; ³The Kinghorn Cancer Centre & Cancer Research Program, Garvan Institute of Medical Research, Darlinghurst; ⁴Department of Anatomical Pathology, South Eastern Area Laboratory Service, St George Hospital, Kogarah; ⁵School of Medicine and Health Sciences, University of Western Sydney, Campbelltown; ⁶School of Medical Sciences and ⁷St Vincent's Clinical School, Faculty of Medicine, University of New South Wales, Kensington; ⁸Department of Tissue Pathology and Diagnostic Oncology, Royal Prince Alfred Hospital, Camperdown; ⁹Central Clinical School, University of Sydney, Sydney, New South Wales, Australia; ¹⁰Royal Marsden Hospital; ¹¹Breakthrough Breast Cancer Research Centre, Institute of Cancer Research, London, United Kingdom; ¹²University of Southern California Norris Comprehensive Cancer Center; and ¹³University of Southern California Center for Molecular Pathways and Drug Discovery, Los Angeles, California

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Corresponding Author: William J. Muller, Goodman Cancer Centre, McGill University, 1160 Pine Avenue, Montreal, QC H3A 1A3, Canada. Phone: 514-398-5847; Fax: 514-398-6769; E-mail: william.muller@mcgill.ca

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cytoplasm and translocation to the nucleus. Nuclear β-catenin forms a complex with T cell-specific transcription factor/lymphoid-enhancer-binding factor (TCF/LEF) and recruits the transcriptional coactivators cAMP-responsive element-binding protein (CREB)-binding protein (CBP) and p300, along with other components of the basal transcription machinery, to regulate the expression of target genes such as *Axin2*, *c-Myc*, and *Tcf7* (8–10). Accumulation of cytosolic and nuclear β-catenin, due to stabilizing mutations in its N-terminal domain, is commonly observed in human cancers such as colorectal carcinoma. Although such mutations are uncommon in human breast cancer, aberrant expression and/or accumulation of β-catenin in the cytoplasm and nucleus have been observed and are associated with poor patient prognosis (11).

In this study, we have used the ErbB2^{KI} model to gain insight into the molecular and genetic events involved in ErbB2-induced mammary tumor progression. Immunohistologic and transcriptional profiling revealed that in contrast to mammary tumors derived from a constitutive ErbB2 mouse model (MMTV/NIC), ErbB2^{KI}-derived tumors expressed markers characteristic of the basal and ERBB2 subtypes of human breast cancer and exhibited activation of the Wnt/β-catenin signaling pathway. Consistent with the ErbB2^{KI} mouse model, a cohort of human ERBB2-positive invasive ductal carcinomas also showed high cytoplasmic β-catenin and abundant expression of basal markers. To further validate the biologic importance of β-catenin signaling in ErbB2-induced tumorigenesis, we used RNA interference to downregulate β-catenin in ErbB2^{KI}-derived mammary tumor cell lines. Reduction of β-catenin levels significantly impaired mammary tumor initiation and metastasis in these tumor cells, and was further associated with reduced expression of components of the *erbB2* amplicon as well as ErbB3. Moreover, treatment of ErbB2^{KI} tumor cells with ICG-001, an inhibitor that specifically antagonizes β-catenin/CBP-mediated transcription, which is critical for maintenance of a nondifferentiated/proliferative state (12, 13), resulted in a proliferative defect, which was accompanied with reduced expression of ErbB2. Similarly, treatment of human ERBB2-positive breast cancer cells with ICG-001 significantly decreased cell proliferation, which correlated with reduced levels of ErbB2. These observations show that targeting of β-catenin-dependent signaling has potential therapeutic value in the treatment of the basal category of ERBB2-positive breast cancer.

Materials and Methods

Gene expression microarray data

Total RNA was isolated from 5 individual ErbB^{KI} tumors. Given the homogeneity of the MMTV/NIC model, 2 RNA pools containing equal amounts of total RNA from 5 individual tumors were generated to obtain MMTV/NIC profiles that are representative of the general population (14). Total RNA isolation and RNA sample preparations were conducted as previously described (14). Labeled aRNA (750 ng) was hybridized against a Universal Mouse Reference RNA (Stratagene) onto Agilent Whole Mouse Genome Oligo Microarrays (G4122A; 44K). Duplicate hybridizations were conducted for all samples using reverse-dye labeling. Microarray data analysis

was conducted in the R statistical programming environment (R Development Core Team, 2008) with Bioconductor (15). Raw and normalized data can be found in National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO; GSE37954). Detailed data management is provided in the Supplementary Data.

qRT-PCR analysis

Total RNA was isolated from flash-frozen mammary tumor tissue using the RNeasy Midi Kit from Qiagen as per the manufacturer's instructions. For quantitative real-time reverse transcription PCR (qRT-PCR) 25 or 50 ng of total RNA were used with the QuantiTect SYBR Green RT-PCR Kit (Qiagen) and the LightCycler (Roche). Each amplification reaction was carried out in triplicate in independent experiments and transcript levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Primer sequences are available as Supplementary Data.

Histology and immunostaining of tissue sections

Mammary tumors and lungs were harvested from mice at tumor endpoint. Tissue was fixed and embedded as described previously (16). Paraffin sections of 5 μm were stained with hematoxylin and eosin (H&E). Step sections of lungs (5 μm × 50 μm) were scanned using a Scanscope XT Digital Slide Scanner (Aperio) and analyzed at 5× magnification using Imagescope software (Aperio) to quantify the total number and surface area of lesions per lung. Immunostaining was conducted as described previously (Supplementary Materials and Methods; ref. 17).

Immunoblotting

Tumor lysates were prepared from flash-frozen tissue and immunoblot analyses were conducted on 15 μg of lysate as described previously (14). For cell lines, extract was prepared in radioimmunoprecipitation assay (RIPA) lysis buffer and 15 μg of lysate was used for immunoblot analyses. Antibodies for immunoblots include: c-Myc, Neu (C18), ErbB3 (C17), and Grb7 from Santa Cruz Biotechnology; β-catenin, survivin, Egfr, and tubulin from Cell Signaling Technology, and β-actin from Sigma-Aldrich. Horseradish peroxidase-conjugated secondary antibodies were obtained from The Jackson Laboratory.

Cell culture, retroviral transduction, and orthotopic transplants

Human breast cancer cell lines [MCF7, SKBR3, HCC202, HCC1954, and MDAMB231 (American Type Culture Collection)] were maintained according to recommendations. ErbB2^{KI}-derived tumor cells (TM15c7-2 and c10-2) were maintained in Dulbecco's Modified Eagle Medium supplemented with 10% FBS and SingleQuots (Clonetics). TM15 cell lines expressing shRNAmirs-targeting β-catenin or nonsilencing shRNA control (Open Biosystems) were generated by retroviral transduction according to the manufacturer's instructions followed by puromycin selection (4 μg/mL; Sigma-Aldrich). For orthotopic transplants, TM15c10-2 cells (1.5 × 10⁵ cells in PBS solution) were injected into the no. 4 inguinal mammary fat pad of athymic nude mice (Taconic). Mice were monitored

twice weekly for tumor formation and tumor growth was assessed by weekly caliper measurements. Mice were sacrificed and tissues were harvested once the tumor volume reached 2,000 mm³. All experiments involving mice were carried out in accordance with McGill University (Montreal, Quebec, Canada) animal care guidelines.

Proliferation assay

CellTiter Aqueous MTS (Promega) proliferation assays were conducted according to the manufacturer's protocols using 2,500 (TM15c10-2 and c7-2) and 5,000 (MMTV/NIC, MCF7, SKBR3, HCC1954, HCC202, and MDAMB231) cells per well (96-well optical-bottom plates, Nunc) in the presence of ICG-001 (10 μmol/L), dimethyl sulfoxide (DMSO), or medium.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) was conducted as described previously (18). Additional details, materials and methods are available in the Supplementary Data.

Human cohort tissues and statistical analysis

The Garvan/St.Vincent's Hospital (Sydney, Australia) outcome series comprises 292 operable invasive ductal carcinomas of the breast from patients treated by a single surgeon between February 1992 and August 2002 at St.Vincent's Hospital. Ethics approval was granted for the use of pathology specimens and cognate clinicopathologic data (Human Research Ethics Committee of St.Vincent's Hospital, Sydney, H00 036). A more detailed description of the clinicopathologic characteristics of the cohort is published elsewhere (Supplementary Materials and Methods; refs. 19, 20). β-Catenin status has previously been determined for this cohort as described previously (Supplementary Materials and Methods; ref. 19).

Definition of intrinsic "molecular" phenotype of breast cancer

This was assessed immunohistochemically using criteria similar to those previously described (21) and FISH to determine ERBB2 status (20). Four different subgroups were defined: basal, ERBB2⁺/ER⁻/PR⁻, luminal A and B. Statistical evaluation was conducted using Statview 5.0 Software (Abacus Systems). A *P* value of less than 0.05 was accepted as statistically significant. ANOVA was used to determine differences in expression of continuous variables across breast cancer subtypes as previously described (22). Kaplan–Meier analysis and Cox-proportional HRs were used to determine association of membrane to cytoplasmic (MTC) score < 0 with breast cancer-specific death (Supplementary Materials and Methods; ref. 19).

Results

ErbB2^{KI} mammary tumors express markers of human basal breast cancer in a heterogeneous fashion

The ErbB2^{KI} model recapitulates many cytogenetic features of human ERBB2-positive breast cancer. On the basis of recent observations that a subset of ERBB2-positive tumors coexpresses ERBB2 and basal cytokeratins (Krts; ref. 23), we investigated the cellular composition of ErbB2^{KI} mammary tumors and compared it with the morphologic features of our con-

ventional MMTV/ErbB2 system (MMTV/NIC). MMTV/NIC transgenic mice coexpress activated ErbB2 and Cre recombinase from the same bicistronic transcript and develop mammary tumors with a phenotype that is comparable with MMTV/NDL2-5 animals, in which only activated ErbB2 is expressed (24). Using cytokeratin markers that are reflective of the different epithelial cell lineages within the mammary gland, we observed uniform expression of the luminal marker cytokeratin 8 in the ErbB2^{KI} tumor (Fig. 1, top). However, many of these tumors retained expression of basal cytokeratin markers such as Krt5, Krt6, and Krt14 (Fig. 1 and Supplementary Fig. S1A). A similar heterogeneous expression was observed for Trp63 and Egfr, which are also associated with a basal phenotype (Fig. 1 and Supplementary Fig. S1A). In contrast, MMTV/NIC tumors were exclusively Krt8-positive but negative for these basal markers (Fig. 1, bottom). These findings suggest that ErbB2^{KI} mammary tumors exhibit a high degree of intratumoral heterogeneity composed of mixed cell lineages, whereas MMTV/NIC tumors are derived from a uniform luminal cell type.

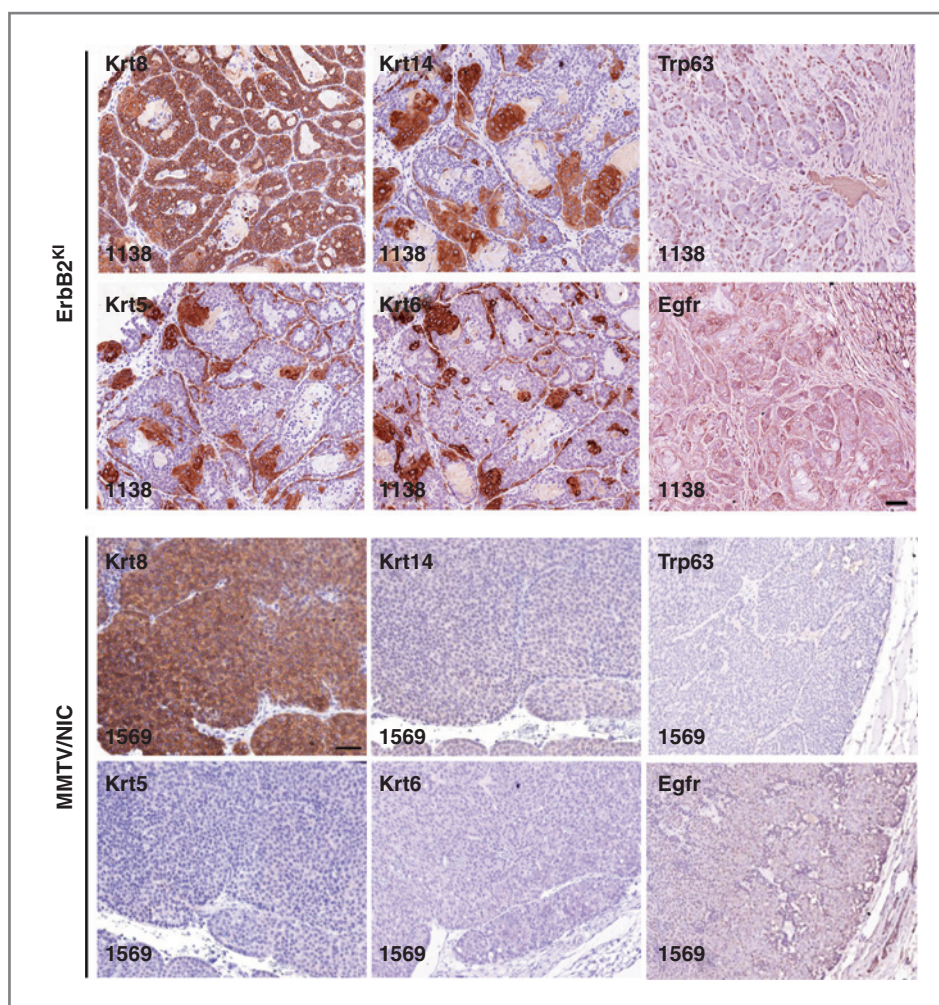
In addition, immunofluorescent staining detected cells coexpressing both cell lineage markers Krt14 and Krt8 in ErbB2^{KI} tumors (Supplementary Fig. S1B). Moreover, tumors with a substantial fraction of Krt8/14–positive cells also showed coexpression of Krt8 and the basal marker Krt5 (Supplementary Fig. S2), suggesting the presence of putative bipotent cells. Taken together, this immunohistopathologic survey indicates that ErbB2^{KI} mammary tumor progression is associated with a high degree of intratumoral heterogeneity.

ErbB2^{KI}-induced mammary tumors possess molecular features of both the ERBB2 and basal-like subtypes of human breast cancer

Previously, we showed that ErbB2^{KI} mammary tumor progression is associated with distinct phenotypical and genomic properties compared with MMTV/ErbB2–derived tumors (25). To further elucidate the molecular basis associated with these unique features of the ErbB2^{KI} model, we obtained transcriptional profiles from ErbB2^{KI}-induced tumors and compared them with profiles from MMTV/NIC tumors and normal mammary glands (FVB m. gl.). Using a class discovery approach with those genes that varied most across the dataset, the samples primarily cluster into 2 defined groups, which completely separate the normal controls (FVB m. gl.) from the tumors (Supplementary Fig. S3A). The analysis further segregates the 2 ErbB2 mouse models into 2 distinct subclusters (Supplementary Fig. S3A). Notably, the ErbB2^{KI} tumor-derived profiles displayed heterogeneous gene expression patterns. Among the transcripts that were upregulated specifically in the ErbB2^{KI} tumors were genes encoding components and targets of the Wnt/β-catenin pathway and the TGF-β signaling pathway (Fig. 2A). Moreover, basal cytokeratins displayed altered transcript levels, supporting our initial immunohistochemical analyses (Fig. 1).

To understand the molecular differences between tumors derived from the 2 ErbB2 models, genes systematically upregulated in each of the models were analyzed for enrichment in Gene Ontology (GO) and Kyoto Encyclopedia of Genes and

Figure 1. ErbB2^{KI} tumors express basal markers. Representative immunostained sections of ErbB2^{KI} tumors with distinct cellular regions positive for basal markers Krt14, 5, and 6, whereas Egfr and Trp63 displayed uniform expression patterns (top). MMTV/NIC tumors were immunonegative for all tested basal markers (bottom). Both ErbB2-induced mouse models abundantly express the luminal marker Krt8. Total number of tumors analyzed: ErbB2^{KI}, *n* = 18; MMTV/NIC, *n* = 10 (scale bar, 50 μm).



Genomes (KEGG) pathways (Supplementary Tables). Although the 2 ErbB2-driven tumor models shared a number of common signaling pathways (Supplementary Tables), there were also some substantial differences. Notably, the ErbB2^{KI} tumors expressed genes related to categories involved in mesenchymal cell development, regulation of the *c-jun*-NH₂-kinase (JNK) cascade, stem cell maintenance, and immune response. In addition, the ErbB2^{KI} tumors preferentially expressed categories of genes involved in Wnt, TGF-β, insulin, and Egfr signaling (Supplementary Tables). In contrast, MMTV/NIC mammary tumors were enriched in KEGG and GO terms related to protein modification, cell growth and death, metabolism, and ErbB and fibroblast growth factor (FGF) receptor signaling (Supplementary Tables). Similar data implicating the Wnt/β-catenin signaling pathway in ErbB2^{KI} tumor progression were obtained using Gene Set Enrichment Analysis (GSEA) analyses, supporting the GO and KEGG results, and particularly highlighting the overrepresentation of Wnt/β-catenin signatures in the ErbB2^{KI} model (Supplementary Tables).

Recently, a murine-specific tumor dataset and intrinsic gene list have been established from transgenic mouse models that resemble the intrinsic subtypes of human breast cancer based

on their gene expression (26). Using this murine-specific intrinsic gene list and tumor dataset, unsupervised hierarchical clustering assigned the ErbB2^{KI} and MMTV/NIC models to distinct molecular groups (Fig. 2B and Supplementary Fig. S3B). MMTV/NIC mammary tumors clustered with another MMTV-driven ErbB2 mouse model (MMTV/Neu) and with other typical luminal-like tumor murine models, including MMTV/polyoma virus middle T antigen tumors (Fig. 2B). In contrast, the ErbB2^{KI} tumors grouped with tumors that are associated with basal characteristics such as MMTV/Cre/BRCA1, p53^{-/-}, p53^{-/+}/insulin receptor, and MMTV/Wnt strains (Fig. 2B; ref. 26).

Next, we explored how the transcriptional profiles generated from 2 different ErbB2 breast cancer mouse models correspond to the human disease. ErbB2 tumor-derived gene expression profiles were compared with profiles from human breast cancers using a cross-species intrinsic gene set as a basis (26). Consistent with our results obtained from the intra-mouse model comparison, the MMTV/NIC tumors clustered with the human luminal subtypes, whereas the ErbB2^{KI} tumors clustered within the human ERBB2 subtype (Fig. 3A). To preclude the influence of the *erbB2* amplicon on human

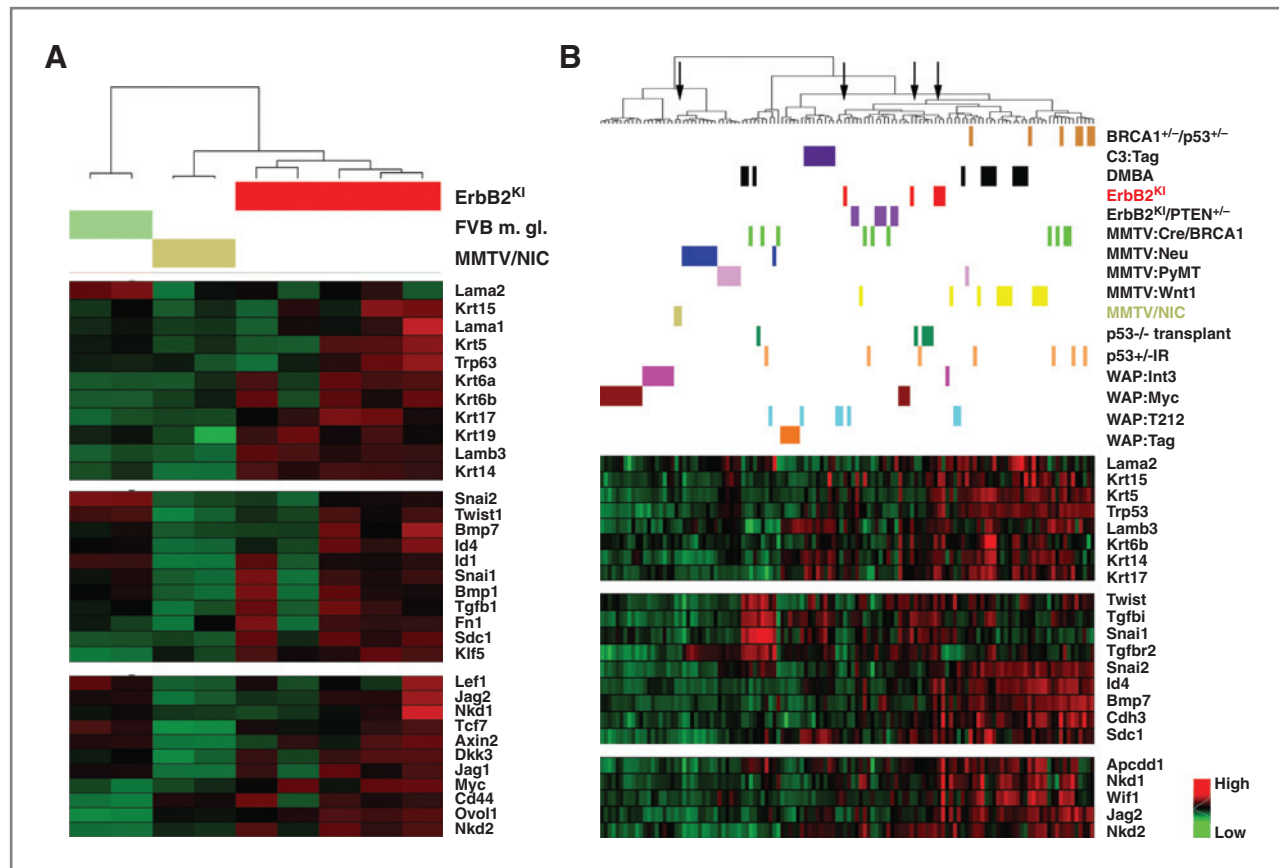


Figure 2. Comparison of ErbB2^{KI} and MMTV/NIC mammary tumors with other transgenic mouse models of breast cancer. A, unsupervised class discovery analysis of normal FVB m. gl., ErbB2^{KI} tumors, and MMTV/NIC tumors; the samples were segregated into 3 separate clusters. B, class discovery analysis of ErbB2^{KI} and MMTV/NIC tumors with various transgenic mouse models of breast cancer using a murine-specific intrinsic gene list. ErbB2^{KI} tumors cluster with mouse models of similar histopathology, whereas MMTV/NIC tumors are associated with luminal-like models. Arrows indicate ErbB2^{KI} tumors or MMTV/NIC tumors within the clusters.

subtypes in this cross-species comparison, we removed *Grb7* (the only *erbB2* amplicon gene found in the original cross-species gene set) from the intrinsic gene list. Although the MMTV/NIC tumors still clustered with the human luminal subtypes, the ErbB2^{KI} tumors now grouped with the basal subtype (Fig. 3B). These findings substantiate the close resemblance of ErbB2^{KI} tumors to the human ERBB2 subtype, but indicate that ErbB2^{KI} mammary tumors contain molecular features of both the ERBB2 and basal subtypes of human breast cancer.

ErbB2^{KI} mammary tumor progression is associated with the activation of the Wnt/ β -catenin signaling cascade

As we had identified a Wnt/ β -catenin signaling axis in ErbB2^{KI} mammary tumors at the transcriptional level (Fig. 2A), we next investigated whether ErbB2^{KI} tumors exhibited evidence of activation of this pathway. To test this possibility, tumor sections from either MMTV/NIC or ErbB2^{KI} mice were subjected to immunofluorescence analyses with antibodies against total β -catenin and activated β -catenin (27). In contrast to the strict membrane localization of total β -catenin and the weak, diffused cytoplasmic signal of the activated form in

the MMTV/NIC tumors, nuclear staining for both forms of β -catenin was detected in 75% (15 of 20) of ErbB2^{KI} samples (Fig. 4A). Consistent with these observations, ErbB2^{KI} mammary tumors with nuclear β -catenin also displayed partial loss of membranous E-cadherin, a protein that forms a complex with β -catenin in adherens junctions, whereas the MMTV/NIC samples showed robust membrane-associated expression of E-cadherin (Fig. 4A). These data suggest that the majority of ErbB2^{KI} tumors show activated Wnt/ β -catenin signaling.

In support of this notion, significant upregulation of direct β -catenin transcriptional targets, including *Cyclin D1*, *Sox9*, *c-Myc*, and 2 hallmarks of an active Wnt/ β -catenin pathway, *Axin2* and *Tcf7*, were detected in most of the ErbB2^{KI} mammary tumors (Fig. 4B–D; refs. 8, 9, 28–30). In contrast, MMTV/NIC tumors showed only basal level expression of these β -catenin targets (Fig. 4B–D). Consistent with the inherent intratumoral heterogeneity observed in ErbB2^{KI} mammary tumors, there was a high degree in variability in the expression of these β -catenin targets within the ErbB2^{KI} cohort.

Cytoplasmic and nuclear β -catenin accumulation has been commonly observed in poor outcome basal-like breast cancers that express ErbB2 (19, 31). In addition, recent studies have

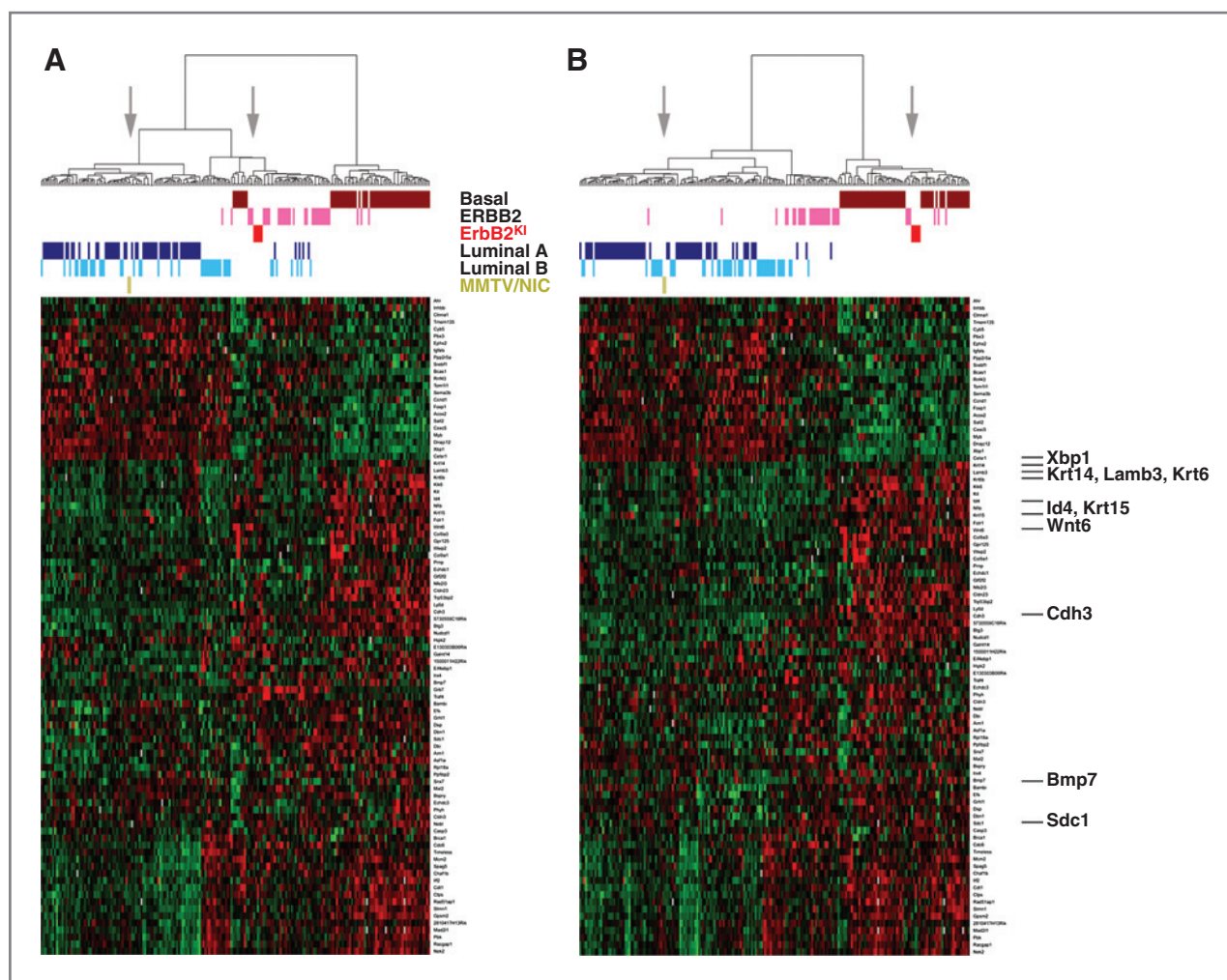


Figure 3. Comparison of mammary tumors from ErbB2 mouse models with primary human breast cancers. Unsupervised hierarchical clustering analyses of ErbB2^{K1} and MMTV/NIC tumors and 181 human breast tumors using genes from the intersection of human and mouse intrinsic gene lists (26). A, the heatmap presented includes one gene (*Grb7*) of the *erbB2* amplicon. The MMTV/NIC tumors cluster with luminal breast cancers, whereas the ErbB2^{K1} tumors group with the ERBB2-positive subtype. B, the cluster analysis was repeated using the mouse-human intrinsic gene list after removing *Grb7*. Although the MMTV/NIC tumors group with luminal breast cancer, the ErbB2^{K1} tumors cluster with the basal subtype. Arrows indicate ErbB2^{K1} tumors or MMTV/NIC tumors within the clusters.

shown that ERBB2-overexpressing tumors can be subdivided in different groups based on their estrogen receptor (ER) status and the expression of basal cytokeratins (23, 32). To establish whether subcellular β-catenin localization impacts on either ER status or the expression of basal cytokeratins, we conducted further statistical analyses of our previously described patient cohort (19). The results showed that tumors with significantly higher cytoplasmic β-catenin [reflected by the MTC score <0 (19)] segregated predominantly to the basal-like and ERBB2⁺/ER⁻/PR⁻ subtypes (Fig. 5A and B). Consistent with the ErbB2^{K1} tumor profiles, both basal and ERBB2⁺/ER⁻/PR⁻ subtypes displayed significantly higher expression of basal cytokeratins (Krt5/6 and Krt14; Fig. 5A and B). Moreover, like the ErbB2^{K1} mammary tumors, partial loss of membranous E-cadherin correlated with high cytoplasmic β-catenin within ERBB2⁺/ER⁻/PR⁻ tumors (Fig. 5A). In addition, ERBB2⁺

/ER⁻/PR⁻ tumors that displayed a cytoplasmic β-catenin expression pattern (MTC score <0; *n* = 16) were associated with a higher rate of breast cancer-specific death (HR, 2.5; *P* = 0.02; Fig. 5C). Thus, our observations indicate that a distinct cohort of ERBB2-positive invasive ductal carcinomas display high cytoplasmic β-catenin, abundant expression of basal markers, partial loss of membranous E-cadherin, and are associated with poor outcome.

Downregulation of β-catenin in ErbB2^{K1}-derived tumor cells impairs both the initiation and metastatic phases of mammary tumor progression

Although the above studies indicate that active β-catenin signaling can be detected in the ErbB2^{K1} tumors and a distinct group of human ERBB2-positive breast cancers, the biologic significance of β-catenin signaling in ERBB2 mammary tumor

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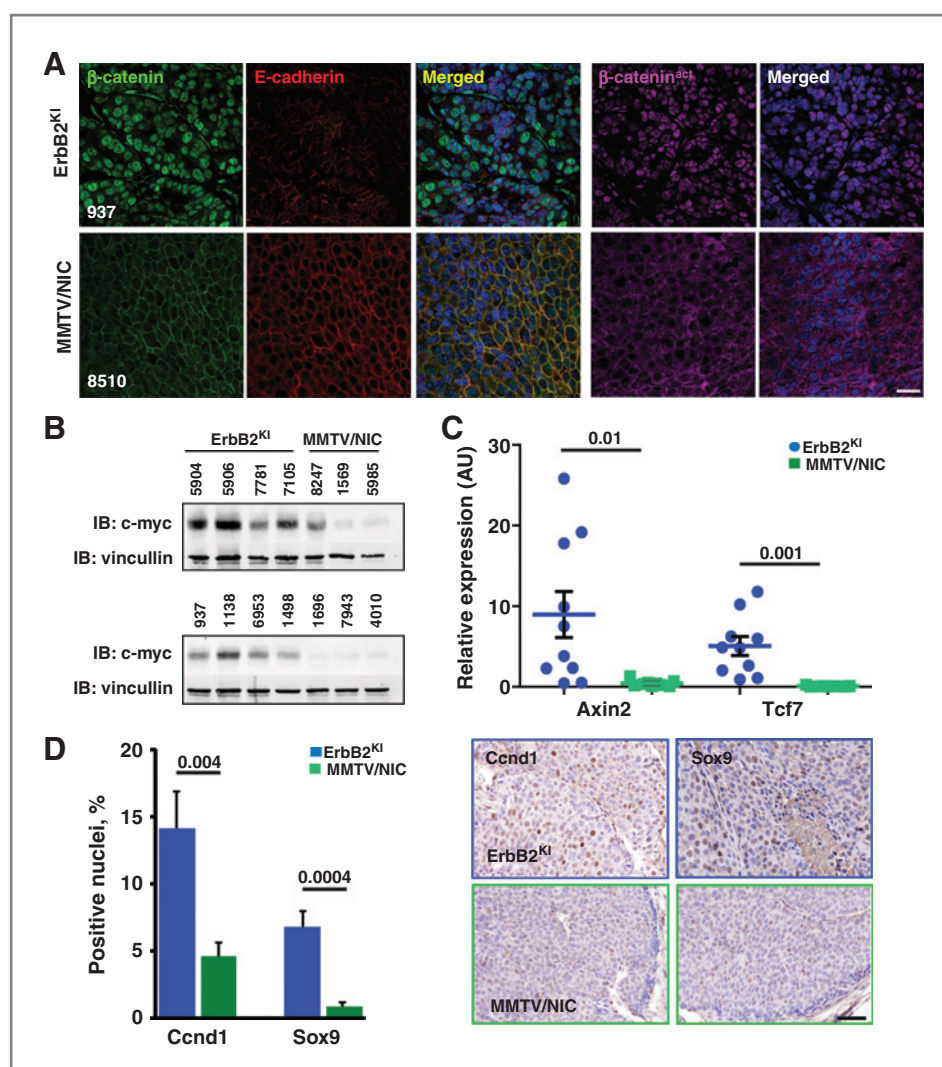


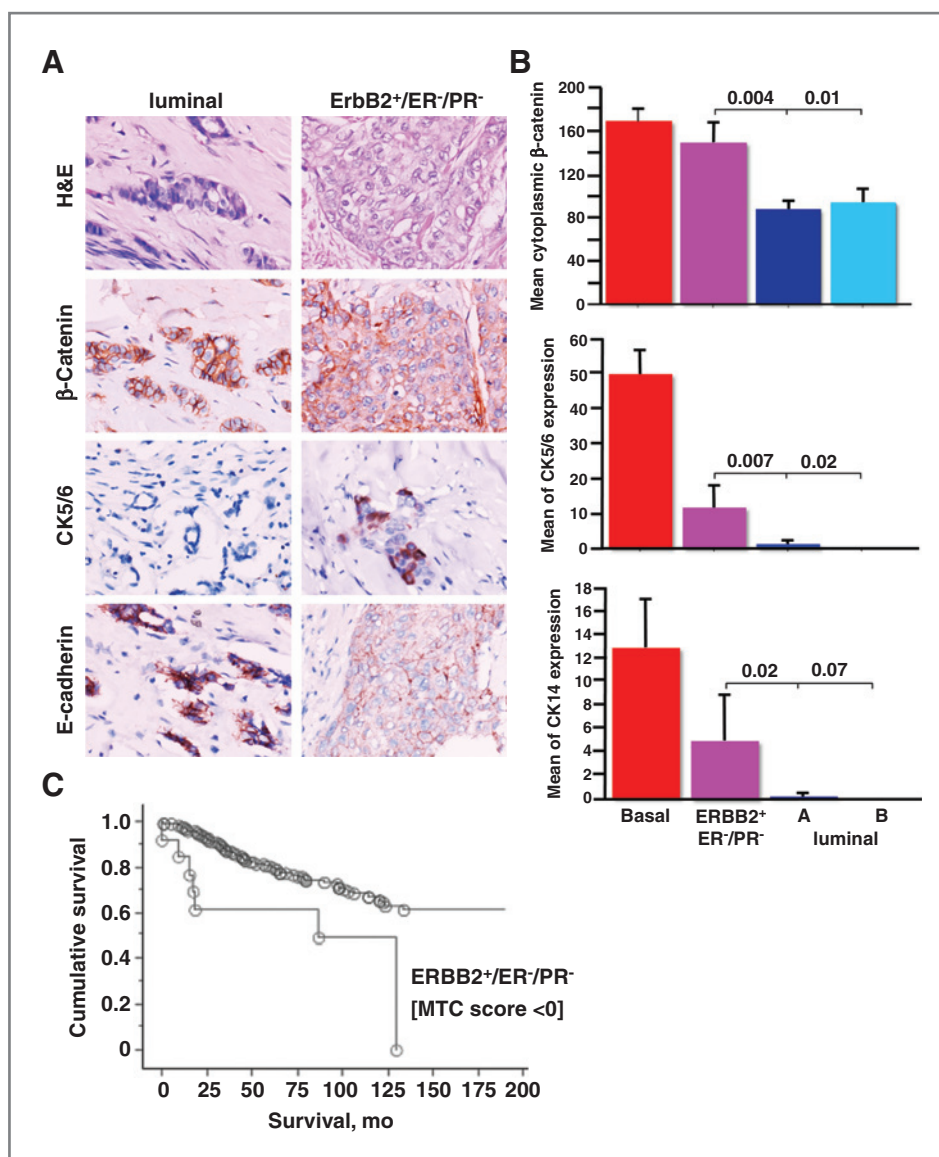
Figure 4. ErbB2^{K1} mammary tumors display Wnt/β-catenin activation. **A**, immunofluorescence analysis of β-catenin (green), E-cadherin (red), and activated β-catenin (purple) in ErbB2^{K1} and MMTV/NIC tumors. Representative sections show nuclear localization for both forms of β-catenin and partial loss of E-cadherin in ErbB2^{K1} tumors ($n = 20$). MMTV/NIC tumors displayed membrane-associated β-catenin and E-cadherin and diffuse cytoplasmic activated β-catenin ($n = 10$). Nuclei were stained with 4',6-diamidino-2-phenylindole (blue; scale bar, 20 μm). **B**, ErbB2^{K1} and MMTV/NIC tumor lysates (15 μg) were subjected to c-myc immunoblot (IB) analysis. Vinculin served as a loading control. **C**, qRT-PCR analysis of Wnt/β-catenin target genes *Axin2* and *Tcf7* as indicated. mRNA expression was normalized to GAPDH. Error bars represent SD of triplicates obtained in 3 independent experiments; P values were calculated by two-tailed t test. **D**, left, data representative of 10 tumors for each ErbB2 mouse model (10–20 fields/tumor) and are depicted as percentage of cyclin D1⁺ (CcnD1) and Sox9⁺ cells \pm SEM. Right, representative images of immunohistochemically stained tumor sections for cyclin D1 and Sox9 (scale bar, 50 μm).

progression is unclear. To directly test the importance of β-catenin in this process, RNA interference was used to down-regulate endogenous β-catenin in ErbB2^{K1}-derived clonal mammary tumor cells. One striking feature of these cell lines is the difference in their ability to metastasize to lung, with 2 clones being highly metastatic (TM15c7-2 and c10-2; ref. 33). To this end, stable c7-2 and c10-2 cell lines expressing either nonspecific shRNA or shRNAs targeting β-catenin (shCttnb1) were generated. Although the *in vitro* growth rates of the pooled clones expressing β-catenin shRNA were comparable with the growth rates of the controls (Supplementary Fig. S4A), the β-catenin-knockdown cells exhibited a pronounced defect in invasion in contrast to the control cells (Supplementary Fig. S4B). Immunoblot analyses confirmed reduced β-catenin protein levels in both cell lines expressing β-catenin-specific shRNAs (Supplementary Fig. S4C and S4D). Interestingly, in addition to the reduced levels of β-catenin, immunoblot analyses also detected a decrease in ErbB2 (Supplementary Fig. S4C and S4D). Similar data were obtained for a different set of β-catenin-specific shRNAs (Supplementary Fig. S4D), indi-

cating that loss of β-catenin results in a reduction in ErbB2 protein levels.

To assess whether β-catenin deficiency could alter the *in vivo* tumorigenic potential of these cells, the tumor cells were injected into the mammary fat pad of athymic mice and monitored for mammary tumor induction. Although control mice exhibited rapid induction of mammary tumors, TM15c10-2 tumor cells expressing the β-catenin shRNA showed a substantial delay in tumor onset (Fig. 6A). In addition to the effect on tumor onset, the TM15c10-2^{shCttnb1} cells displayed striking differences in their metastatic potential when compared with controls. Although 100% of the control animals developed lung metastases with an average number of 15 to 16 lesions per lung, only 60% of mice injected with TM15c10-2^{shCttnb1} cells formed lung lesions, with one lesion per lung on average (Fig. 6B). Similarly, the average total surface area of these lesions was dramatically reduced in the mice injected with the β-catenin-deficient tumor cells when compared with surface area of lesions in the control groups (Supplementary Fig. S5A).

Figure 5. Human ERBB2 "molecular" breast cancer subtypes (ERBB2⁺/ER⁻/PR⁻) show high expression of cytoplasmic β-catenin and basal marker cytokeratin 5/6. A, immunohistochemical analysis of luminal and ERBB2⁺/ER⁻/PR⁻ breast tumors; representative H&E-, β-catenin-, cytokeratin 5/6 (CK5/6)-, and E-cadherin-stained sections. Images were taken at ×400. B, basal-like and ERBB2⁺/ER⁻/PR⁻ subtypes expressed the highest levels of cytoplasmic β-catenin, CK5/6, and CK14 when compared with luminal A and B subtypes. C, Kaplan–Meier survival analysis of the breast cancer patient cohort showing separation of patients of the ERBB2⁺/ER⁻/PR⁻ subtype that have cytoplasmic β-catenin expression as indicated (n = 16; MTC score <0). These patients had a worse outcome in comparison with the rest of the cohort of invasive ductal carcinoma.



Because β-catenin-deficient ErbB2^{K1} cells exhibited low levels of ErbB2 protein (Supplementary Fig. S4C and S4D), we next examined the expression of ErbB2 in mammary fat pad outgrowths of ErbB2^{K1} cell lines lacking β-catenin and control groups. Consistent with our *in vitro* studies, β-catenin-deficient tumors showed a significant decrease in ErbB2 protein and transcript levels, whereas relative *erbB2* amplification was unchanged across all samples (Fig. 6C and Supplementary Fig. S5B). Moreover, given the strong correlation between the expression of ErbB2, ErbB3, and components of the *erbB2* amplicon, including Grb7, Stard3, and Perld1, we assessed their expression in these samples (34, 35). Like ErbB2 levels, the expression of these proteins was significantly reduced in β-catenin-deficient mammary tumors (Fig. 6C).

To verify that β-catenin signaling was impaired in these tumors, the relative expression of several β-catenin target

genes was measured by qRT-PCR analysis. Indeed, significant decrease in β-catenin, *Axin2*, and *Tcf7* transcripts were detected in most of the β-catenin-deficient tumors, as well as reduced expression of *Cyclin D1* (Supplementary Fig. S5C and S5D). These observations indicate that β-catenin is critical for both the initiation and metastatic phases of ErbB2^{K1} mammary tumor progression.

Inhibition of β-catenin/CBP-dependent signaling by ICG-001 decreases tumor cell proliferation and leads to reduced ErbB2/ERBB2 expression in ErbB2^{K1} mouse and ERBB2-positive human mammary tumor cells

To further address the role of β-catenin signaling in mammary tumorigenesis, we next assessed whether administration of a small-molecule inhibitor of β-catenin (ICG-001) would impact on the transformed phenotype of ErbB2-driven tumor cells. ICG-001 specifically prevents β-catenin/CBP-mediated

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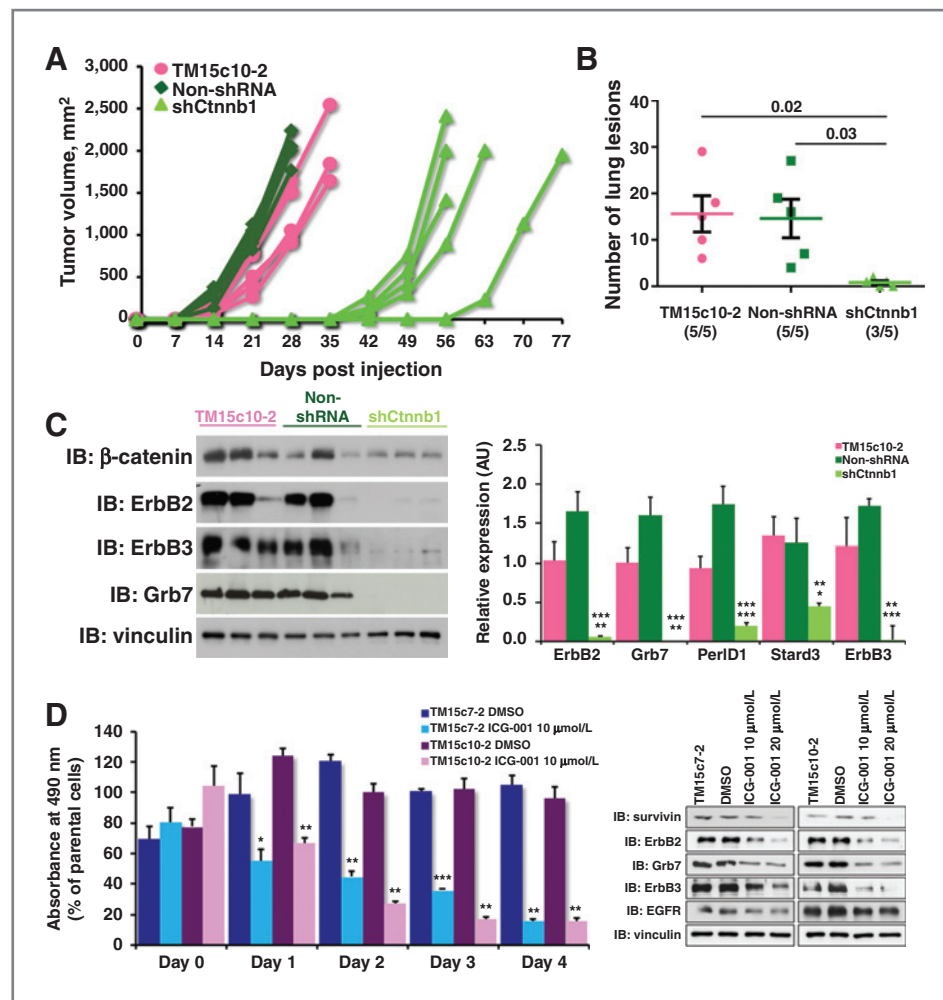


Figure 6. Inhibition of β -catenin signaling in ErbB2^{KI}-derived tumor cells impairs tumor initiation and metastasis and is associated with reduced expression of *erbB2* amplicon components as well as ErbB3. **A**, ErbB2^{KI} tumor cells (TM15c10-2) and stable cells expressing nonsilencing shRNA control (non-shRNA) or β -catenin-specific shRNA (shCtnnb1) were injected into the mammary fat pad of athymic mice and tissue was harvested at endpoint (2,000 mm³). Tumor volumes are presented for 5 independent mice for each group. **B**, H&E-stained step sections of lungs were scored for the total number of lesions per lung as indicated. Ratios in parentheses indicate the number of mice with lung lesions relative to the total number of mice examined. Error bars represent SEM (two-tailed *t* test). **C**, left, tumor lysates (15 μ g) from each group were immunoblotted for the indicated proteins. Right, mean relative mRNA expression levels of Grb7, Stard3, PeriD1, ErbB2, and ErbB3 transcript measured by qRT-PCR in TM15c10-2, non-shRNA, and shCtnnb1 tumors as indicated. mRNA levels were normalized to GAPDH. Error bars represent SD of triplicates obtained in 3 independent experiments. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; two-tailed *t* test versus controls. **D**, left, graph showing the proliferation of ErbB2^{KI} cells (TM15c7-2, c10-2) treated with ICG-001 as indicated (MTS proliferation assay). Data are normalized to values from parental cells. Error bars represent SEM of triplicates obtained in independent experiments; *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; two-tailed *t* test versus controls. Right, ErbB2^{KI} cell lysates (15 μ g) were treated with ICG-001 for 48 hours and subjected to immunoblot (IB) analysis for the indicated proteins.

transcription, which is critical for maintenance of a nondifferentiated/proliferative state, without affecting β -catenin/p300-dependent signaling, which is important for initiation of cellular differentiation (12, 13). Treatment of 2 independent ErbB2^{KI}-derived tumor cell lines (TM15c7-2 and c10-2) with ICG-001 resulted in a clear proliferative defect in both cell lines without impacting the apoptotic status of the cells (Fig. 6D, left). In contrast, proliferation in 2 independent MMTV/NIC-derived tumor cell lines was not affected by ICG-001 treatment (Supplementary Fig. S5E), supporting our data that β -catenin signaling is not activated in this MMTV-driven ErbB2 mouse model.

Biochemical analyses of ICG-001-treated ErbB2^{KI} cells revealed lower levels of survivin, a β -catenin/CBP-regulated target gene (36), in these cells (Fig. 6D, right). Like β -catenin-deficient ErbB2^{KI} tumor cells, specific inhibition of the β -catenin/CBP signaling resulted in reduced expression of ErbB2, Egfr, Grb7, and ErbB3 (Fig. 6D, right). Collectively, these observations argue that inhibition of β -catenin/CBP function severely affected ErbB2^{KI} tumor cell proliferation accompanied by a reduction in the levels of Egfr family members (ErbB2, ErbB3, and Egfr) and Grb7.

To confirm the importance of β -catenin/CBP signaling in human ERBB2-dependent breast tumor progression, we

evaluated whether ICG-001 impacts on the proliferative status of a number of ERBB2-expressing human breast cancer cell lines. Given the association of β-catenin signaling with ERBB2-overexpressing breast cancer cells that are ER⁻/PR⁻, we evaluated the β-catenin status in 3 such lines (HCC202, HCC1954, and SKBR3; ref. 37). Immunofluorescence analysis using β-catenin and activated β-catenin antibodies revealed the presence of activated β-catenin in the nucleus in all 3 ERBB2⁺/ER⁻/PR⁻ cell lines (Fig. 7A). In contrast, luminal MCF7 cells (ERBB2⁻/ER⁺/PR⁺) exhibited strict membrane localization and the classical basal cell line MDAMB231 (ER⁻/PR⁻/ERBB2⁻) showed nuclear localization of β-catenin (Supplementary Fig. S6A). We next assessed the effects of inhibition of β-catenin/CBP-mediated transcription by incubating these human breast cancer cells with ICG-001. The results showed that all cell lines with evidence of activated β-catenin exhibited either a strong (SKBR3 and MDAMB231) or a moderate (HCC202 and HCC1954) growth inhibition (Fig. 7B and Supplementary Fig. S6B). In contrast, ICG-001 had a modest effect on cell proliferation in the luminal control cancer cell line (MCF7; Supplementary Fig. S6B). The observed ICG-001-mediated proliferative defect was not associated with an increase in apoptosis as cell surface Annexin V staining was unaffected (Supplementary Fig. S6C).

Consistent with impaired β-catenin/CBP signaling, biochemical analyses of ICG-001-treated tumor cells in comparison with DMSO- and untreated cells exhibited significantly reduced levels of survivin in the basal and all 3 ERBB2-positive cell lines (Fig. 7C and Supplementary Fig. S6D). Moreover, in support with our data obtained from ErbB2^{K1} tumor cells, ERBB2-overexpressing human cell lines displayed substantially lower levels of ERBB2, GRB7, ERBB3, and EGF receptor (EGFR) when treated with ICG-001 (Fig. 7C). Together, these data suggest that targeting β-catenin/CBP signaling results in the repression of ERBB2 and an associated decrease in cell proliferation among ERBB2-overexpressing breast cancer cell lines.

To this end, our data indicate that antagonizing β-catenin signaling leads to repression of ErbB2/ERBB2 expression in both mouse and human mammary tumor cells. To explore whether β-catenin directly activated *ERBB2* transcription, we conducted ChIP analyses on the *ERBB2* promoter with β-catenin- and RNA polymerase II-specific antisera. Although we could not detect recruitment of β-catenin to the *ERBB2* proximal promoter, we detected a 3-fold enrichment in β-catenin and a 20-fold enrichment in RNA polymerase II recruitment to an intronic *ERBB2* site (Fig. 7D), which has been shown to be bound by several factors that are important in the transcriptional regulation of *ERBB2* (18, 38). β-Catenin and RNA polymerase II occupancy to this site was further enriched upon treatment with the Wnt3 ligand (Fig. 7D). We further exposed the cells to ICG-001 for 48 hours and there was no impact on recruitment of β-catenin to the intronic *ERBB2* site (data not shown), whereas RNA polymerase II occupancy was substantially decreased (Fig. 7D). Importantly, activation by Wnt/β-catenin signaling in SKBR3 cells led to a 2-fold increase in the recruitment of RNA polymerase II to the promoter of *ERBB2*, whereas inhibition of β-catenin/CBP by ICG-001 decreased RNA

polymerase II recruitment by 2-fold (Fig. 7D). Interestingly, we observed a similar impact on RNA polymerase II occupancy on the proximal promoter of other components of the *ERBB2* amplicon after Wnt3 and ICG-001 treatment (Grb7, Stard3, and PerlD1; Supplementary Fig. S6E). Together, these data indicate that β-catenin/CBP-mediated signaling directly regulates *ERBB2* expression in ERBB2-positive breast cancer cells.

Discussion

A hallmark of human breast cancer is its intrinsic heterogeneity, reflecting the underlying molecular complexity of the disease. On the basis of comprehensive gene expression profiling, 5 major molecular subtypes have been identified (luminal A, luminal B, basal-like, ERBB2-positive, and normal-like) that are associated with differences in patient outcome and response to treatment (39). A growing body of evidence established by transcriptional profiling, comparative genome hybridization, and immunohistochemical analyses, suggests that the biology of ERBB2-positive tumors is similarly heterogeneous. Indeed, this molecular subtype can be subdivided into ER⁺ and ER⁻ tumors, which may explain the variable response to ERBB2-targeted therapy (23, 32, 40–43). To address this issue, we used the ErbB2^{K1} mouse model, which exhibits several unique features, including expression of ErbB2 from its endogenous promoter and spontaneous amplification of the *erbB2* locus, resembling that of human ERBB2-positive breast cancers (4). In this study, we showed that ErbB2^{K1} mammary tumor progression is associated with heterogeneous expression of distinct basal (Krt5, Krt6, Trp63, and Egfr), myoepithelial (Krt14), and luminal markers (Krt8), and the presence of cells exhibiting bipotential characteristics, whereas constitutive MMTV/NIC tumors are composed of a uniform luminal epithelial cell type (Fig. 1 and Supplementary Figs. S1 and S2).

Consistent with these histopathologic features, the gene expression signatures from ErbB2^{K1} mammary tumors differ from those of MMTV/NIC tumors (Fig. 2A). Although genes related to protein modification, cell growth, and death, and FGF receptor signaling were associated with MMTV/NIC tumors, the transcriptional signature obtained from ErbB2^{K1} tumors comprised genes implicated in mesenchymal cell development, Wnt, TGF-β, and Egfr signaling pathways (Fig. 2A and Supplementary Tables). Moreover, a comparison of these ErbB2-driven expression profiles with data derived from other murine models revealed that ErbB2^{K1} samples shared molecular features with murine models representative of the mesenchymal/basal phenotype (Fig. 2B). Using an intrinsic mouse/human signature (26), we further showed that ErbB2^{K1} mammary tumors closely resemble both the basal-like and ERBB2-positive subtypes of human breast cancer (Fig. 3). In contrast, MMTV/NIC tumors exhibited transcriptional features typical of the solid/luminal subtype (Fig. 3). Because high ErbB2 expression is achieved much earlier in the MMTV/NIC strain in comparison with ErbB2^{K1} mice, it is likely that the tumor-initiating cell type may differ between the 2 models. In support of this argument, ErbB2^{K1} mammary tumors contain cells expressing Trp63 and coexpressing Krt8/14 and Krt8/5

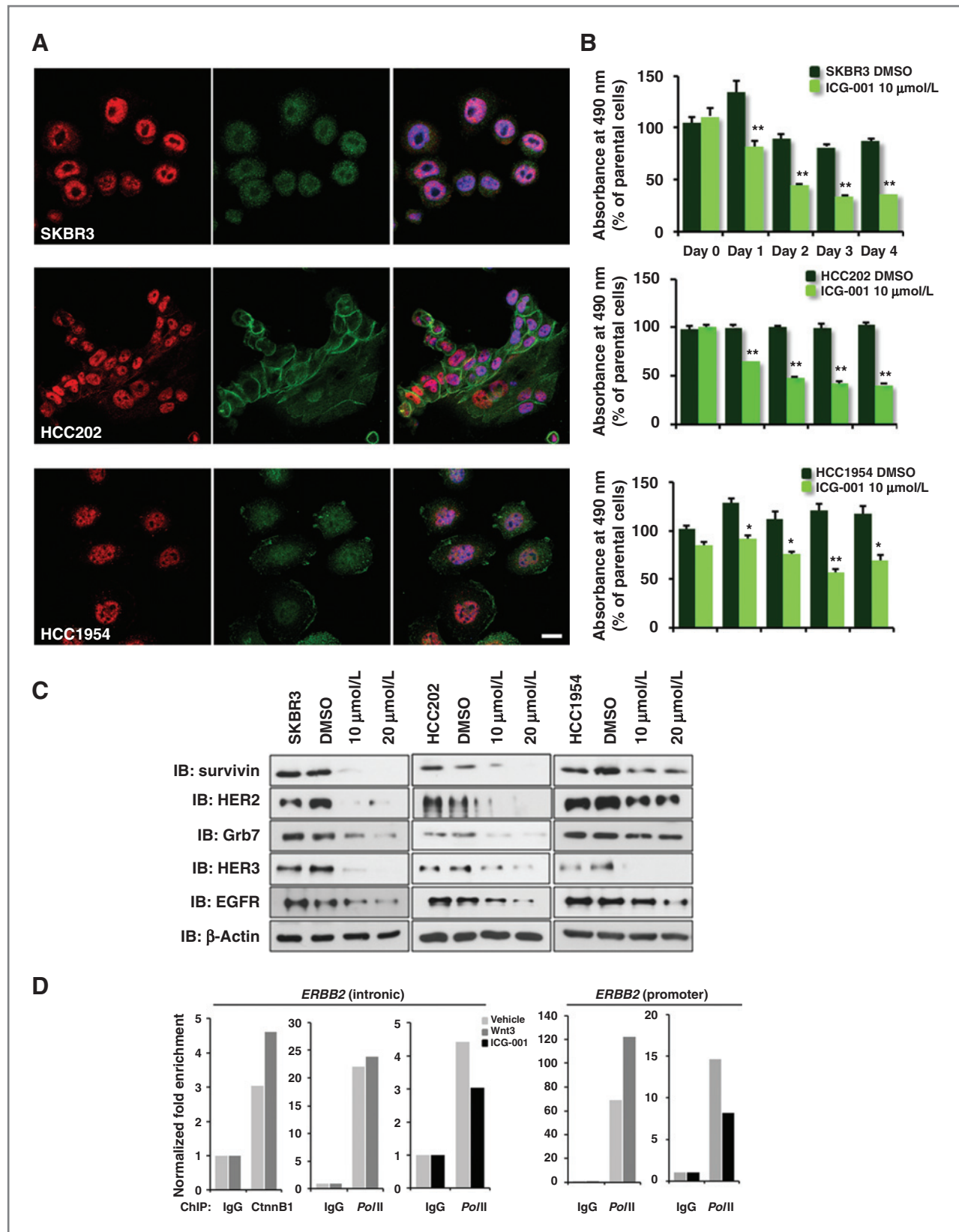


Figure 7. Inhibition of β -catenin/CBP function in human ERBB2-overexpressing breast cancer cells leads to a proliferative defect accompanied by reduced ErbB2 and ErbB3 protein. A, immunofluorescence staining was used to visualize localization of β -catenin (green) and activated β -catenin (red) in human

(Fig. 1 and Supplementary Figs. S1 and S2), which are indicative of a multipotent phenotype (44, 45).

On the basis of our gene expression data, we found that ErbB2^{K1} mammary tumors overexpressed components of the Wnt/β-catenin signaling pathway, contained cellular regions with nuclear β-catenin, and abundantly expressed classical Wnt/β-catenin targets, including cyclin D1, c-Myc, Axin2, and Tcf7 (Figs. 2 and 4). These findings indicate that ErbB2^{K1} mammary tumor progression is associated with Wnt/β-catenin activation. Consistent with this concept, recent genomic and immunohistochemical profiling of human ERBB2-amplified tumors identified an ERBB2⁺/ER⁻ subgroup that was associated with the accumulation of cytoplasmic β-catenin, overexpression of Wnt/β-catenin signaling components, and increased risk of recurrence (41). We identified a subgroup within our ERBB2-positive patient cohort characterized as ERBB2⁺/ER⁻/PR⁻, which predominantly displayed cytoplasmic β-catenin and was associated with poor clinical outcome (Fig. 5). Collectively, these observations suggest that mammary tumor progression in the ErbB2^{K1} mouse model recapitulates many of the molecular events observed in the human basal-ERBB2 subtype, including activation of a β-catenin signaling network.

The importance of β-catenin signaling in ErbB2 mammary tumorigenesis is further highlighted by the observation that downregulation of endogenous β-catenin expression in ErbB2^{K1}-derived mammary tumor cells (TM15c7-2 and c10-2) severely impaired the invasive capacity of these cells *in vitro* (Supplementary Fig. S4) and impacted both the initiation and metastatic phases of tumor progression *in vivo* (Fig. 6). Our observations are consistent with numerous reports where genetic ablation of β-catenin in various *in vivo* systems diminished metastatic progression (46, 47), providing support for β-catenin as a critical mediator of the metastatic process.

The importance of β-catenin in ErbB2 tumor induction is further supported by studies with the ICG-001 small-molecule inhibitor that specifically antagonizes the interaction of β-catenin with CBP but not with p300, facilitating downregulation of a subset of β-catenin/CBP-responsive genes. Treatment with ICG-001 caused a significant proliferative defect in mouse ErbB2^{K1} mammary tumor cells and in several human ERBB2-overexpressing cancer cells *in vitro*. Both cell systems showed reduced levels of survivin, which is indicative of antagonized β-catenin/CBP signaling (Figs. 6D and 7C). This growth defect was not related to increased apoptosis but rather associated with a defect in cell proliferation. A body of evidence indicates that survivin function is not only limited to inhibition of apoptosis but also involves the regulation of cell division; its overexpression allows cancer cells to resume cell division (reviewed in ref. 48).

Interestingly, while inhibition of β-catenin/CBP signaling resulted in a proliferative defect, downregulation of endogenous β-catenin in ErbB2^{K1}-derived tumor cells affected invasion but not cell growth. One possible explanation for this difference is that ICG-001 antagonizes the expression of a specific subset of β-catenin/CBP-responsive genes, whereas downregulation of β-catenin could lead to a wide range of effects. Consistent with the importance of β-catenin in cellular viability, stable cell lines expressing β-catenin-specific shRNAs still retained basal level expression of β-catenin (Fig. 6C and Supplementary Fig. S4C and S4D). Given the ability of β-catenin to recruit histone remodeling complexes such as SWI/SNF and polycomb repressor complexes 1 and 2 (PCR1/2; refs. 49–51), it is conceivable that downregulation of β-catenin in ErbB2^{K1} tumor cells results in genome-wide reprogramming due to an altered epigenetic landscape, which compensates for loss of β-catenin. As a consequence, these cells maintained their proliferative properties but lost their invasive potential. Further experiments are needed to enhance our understanding of β-catenin signal integration and cross-talk in ErbB2-overexpressing tumor cells.

The complexity by which β-catenin regulates Wnt-dependent and -independent transcription has only emerged recently (reviewed in ref. 52). Aberrant activation of the Wnt pathway leads to nuclear localization of β-catenin where it functions as a scaffold to link TCF factors with numerous complexes to regulate target genes. In this study, we have found that loss of endogenous β-catenin in mouse ErbB2^{K1} tumor cells correlated with low expression of ErbB2, Grb7, Stard3, Per1D1, and ErbB3 (Fig. 6C). Similarly, inhibition of β-catenin/CBP signaling by ICG-001 in this system significantly suppresses ERBB2, Grb7, and ERBB3 expression in a panel of human ERBB2-overexpressing tumor cells (Figs. 6D and 7C). Moreover, our ChIP data also illustrate a possible regulation of *ERBB2* gene expression by β-catenin/CBP signaling (Fig. 7D). We found recruitment of β-catenin and RNA polymerase II at an intronic *ERBB2* site, which was further enhanced upon Wnt3 exposure, whereas ICG-001 treatment disrupted RNA polymerase II occupancy at this site. This site is particularly important as transcription factors such as ERRα and ER-α have been shown to compete for binding to this site to activate or repress *ERBB2* transcription (18, 38). In addition, recent data have established that ERRα, β-catenin, and Lef1 form a transcriptionally active complex to activate gene expression (53). Taken together, the strong reduction of ERBB2 expression observed upon ablation of β-catenin activity either through RNA interference or ICG-001 is due to a disruption of the ERRα/β-catenin/CBP-dependent recruitment of RNA polymerase II to an intronic *ERBB2* site that regulates *ERBB2* transcription. Consistent with these data, it has been previously shown that ICG-001 specifically

ERBB2-overexpressing ER⁻ tumor cells (SKBR3, HCC202, HCC1954; scale bar, 20 μmol/L). B, MTS proliferation assays were conducted on ICG-001-treated and control human breast tumor cells as indicated. Data are normalized to values from parental cells. Error bars represent SEM of triplicates from independent experiments; *, *P* < 0.01; **, *P* < 0.001; two-tailed *t* test versus DMSO control). C, ICG-001-treated tumor cells (48 hours) and relevant controls were lysed and immunoblotted (IB) for the indicated proteins (15 μg of lysates). D, standard ChIP experiment in SKBR3 cells shows β-catenin and RNA-PolII recruitment to the intronic *ERBB2* site. Wnt3 exposure (100 ng/mL; 4 hours) increased β-catenin and RNA-PolII recruitment. ICG-001 (10 μmol/L; 48 hours) decreased RNA-PolII recruitment to the intronic *ERBB2* site. Wnt3 increased, whereas ICG-001 decreased RNA-PolII occupancy on the *ERBB2* promoter. Values were normalized against the control region and against the immunoglobulin G (IgG) control.

interferes with the ability of β -catenin to form complexes with CBP and Lef1 (54). The ICG-001-dependent reduction in *GRB7* transcription can also be attributed to the fact that the proximal promoters of *GRB7* and other *ERBB2* amplicon components are also regulated by these β -catenin-containing complexes (Supplementary Fig. S6D and S6E).

Collectively, this work highlights the relevance of the ErbB2^{K1} mouse strain as a preclinical model system of ERBB2-positive breast cancer and provides novel insights into β -catenin signaling in ERBB2-mediated breast cancer progression. These data further implicate β -catenin as a potential target in ERBB2-positive breast cancer and show how the inhibition of a specific function of β -catenin presents a novel therapeutic approach that could improve the outcome of patients with this disease.

Disclosure of Potential Conflicts of Interest

S.A. O'Toole has Commercial Research Grant from Novartis, honoraria from Speakers Bureau of Lilly Oncology, and is a consultant/advisory board member of Roche. M. Kahn has ownership interest (including patents) in Prism BioLab and is a consultant/advisory board member of Prism BioLab. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: B. Schade, W.J. Muller

Development of methodology: B. Schade, V. Sanguin-Gendreau, V. Giguère, M. Kahn, W.J. Muller

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B. Schade, V. Sanguin-Gendreau, T. Bui, G. Deblois, S.A. O'Toole, E.K.A. Millar, S.J. Zardawi, E. Lopez-Knowles, R.L. Sutherland, V. Giguère, W.J. Muller

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