

A NIK-IKK α Module Expands ErbB2-Induced Tumor-Initiating Cells by Stimulating Nuclear Export of p27/Kip1

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<http://dx.doi.org/10.1016/j.ccr.2013.03.012>

SUMMARY

I κ B kinase α (IKK α) activity is required for ErbB2-induced mammary tumorigenesis. Here, we show that IKK α and its activator, NF- κ B-inducing kinase (NIK), support the expansion of tumor-initiating cells (TICs) that copurify with a CD24^{med}CD49^{hi} population from premalignant ErbB2-expressing mammary glands. Upon activation, IKK α enters the nucleus, phosphorylates the cyclin-dependent kinase (CDK) inhibitor p27/Kip1, and stimulates its nuclear export or exclusion. Reduced p27 expression rescues mammary tumorigenesis in mice deficient in IKK α kinase activity and restores TIC self-renewal. IKK α is also likely to be involved in human breast cancer, where its expression shows an inverse correlation with metastasis-free survival, and its presence in the nucleus of invasive ductal carcinomas (IDCs) is associated with decreased nuclear p27 abundance.

INTRODUCTION

Breast cancer (BCa), the leading cause of cancer deaths in women (Jemal et al., 2011), can be classified into Luminal A, Luminal B, ERBB2/HER2 positive, triple-negative, and normal types based on estrogen receptor (ER), progesterone receptor (PR), and ERBB2 expression. Genomic amplification of the *ERBB2* locus and/or overexpression of its product occur in 20%–30% of BCa and correlate with poor prognosis (Borg et al., 1989). Trastuzumab, a humanized monoclonal ERBB2 antibody, is effective in treating ERBB2-positive BCa. However, most patients develop resistance to such drugs, necessitating identification of new therapeutic strategies that target tumor and metastasis initiating cells.

The tumor-initiating cells (TICs) of ERBB2-induced breast and mammary cancers remain elusive. Within normal and lactating

mammary glands, the mouse mammary epithelium consists of CD24^{hi}CD49^{lo}CD29^{lo} lineage-free (L⁻) luminal cells, CD24^{med}CD49⁺CD29^{hi}L⁻ basal cells, and alveolar cells (Shackleton et al., 2006; Stingl et al., 2006). Luminal epithelial cells comprise CD61⁺ luminal progenitors and CD61⁻ mature luminal cells (Asselin-Labat et al., 2007), whereas basal epithelial cells include CD24^{med}CD49^{hi} cells that are enriched for mammary stem cells (MaSC) and CD24^{med}CD49^{lo} myoepithelial cells (Shackleton et al., 2006; Stingl et al., 2006). ErbB2-induced mammary cancer was suggested to be initiated by a subpopulation of parity-identified mammary epithelial cells (PI-MECs) within CD24^{hi}CD49^{lo}L⁻ luminal cells, whose proliferation is driven by cyclin D1. CD24^{med}CD49⁺L⁻ basal cells that are enriched for MaSCs, however, are not regulated by cyclin D1 (Jesselsohn et al., 2010). Ablation of cyclin D1 in the mammary epithelium retards lobuloalveolar development during

Significance

Our results indicate that the NIK-IKK α module is an important regulator of ErbB2-induced mammary tumorigenesis by virtue of its ability to promote the nuclear exclusion of p27/Kip1, thereby supporting the proliferation and expansion of TICs. Although the IKK α -dependent TICs copurify with a cell population that expresses basal cell markers, these cells give rise to luminal tumors. The presence of nuclear IKK α in human breast cancer and its correlation with the nuclear exclusion of p27 suggest that targeting the NIK-IKK α module offers an opportunity to prevent the expansion of breast cancer TICs and thereby control tumor recurrence and metastatic spread.

pregnancy and causes defective lactation (Fantl et al., 1995). A similar phenotype is exhibited by females homozygous for an *Ikk α ^{AA}* knockin allele, in which I κ B kinase α (IKK α) activation is prevented by replacement of activation loop serines with alanines (Cao et al., 2001). Furthermore, IKK α activity is required for induction of cyclin D1 upon engagement of receptor activator of NF- κ B (RANK) during pregnancy (Cao et al., 2001). RANK activation by RANK ligand (RANKL) produced by PR⁺ luminal cells drives progesterone-induced basal MaSC expansion (Asselin-Labat et al., 2010; Joshi et al., 2010) and mammary tumorigenesis (Gonzalez-Suarez et al., 2010; Schramek et al., 2010). However, in the absence of progesterone, RANKL is produced by tumor-infiltrating FoxP3⁺ T cells that cause the IKK α -dependent metastatic spread of mammary cancer cells (Tan et al., 2011). Enhanced RANK expression is also associated with increased metastasis in human BCa (Palafox et al., 2012). *MMTV-ErbB2* mice that are homozygote for the *Ikk α ^{AA}* allele exhibit delayed tumorigenesis, due to defective TIC self-renewal, but this defect appears to be cyclin D1 independent (Cao et al., 2007).

Besides cyclin D1, other regulators of G1-S phase transition, including CDK2 (Ray et al., 2011), CDK4 (Yu et al., 2006), and p27/Kip1 (Hulit et al., 2006; Muraoka et al., 2002), also participate in ErbB2-induced mammary tumorigenesis. p27 binds cyclin E/A:CDK2 complexes to inhibit G1-S transition (Lu and Hunter, 2010). *Kip1* is a haploinsufficient suppressor of ErbB2-induced mammary tumorigenesis; *MMTV-ErbB2/Kip1^{+/-}* female mice develop mammary tumors earlier than *MMTV-ErbB2/Kip1^{+/+}* mice, but complete *Kip1* deficiency prevents ErbB2-induced mammary tumorigenesis (Muraoka et al., 2002). *Kip1* is rarely inactivated in human cancers, although reduced p27 expression (Catzavelos et al., 1997; Porter et al., 1997) and nuclear exclusion (Shin et al., 2002; Viglietto et al., 2002) correlate with poor prognosis. Tumor promotion by decreased p27-mediated cyclin-dependent kinase (CDK) inhibition may be due to expansion of stem/progenitor cells (Besson et al., 2007), consistent with observations that p27 plays an important role in self-renewal of human embryonic stem cells (Menchón et al., 2011). Several protein kinases that phosphorylate p27 and induce its nuclear export were previously described (Lu and Hunter, 2010).

Our previous study demonstrates that inactivation of IKK α led to lowered incidence and delayed onset, but not complete inhibition, of mammary tumorigenesis (Cao et al., 2007) in *MMTV-ErbB2* Tg mice, suggesting ErbB2-induced mammary tumorigenesis may originate from IKK α -dependent and IKK α -independent TICs. In our current study, we aim to identify TICs for ErbB2-induced mammary tumorigenesis and also examine the role of IKK α and its related signaling pathway in regulating mammary TICs.

RESULTS

ErbB2-Induced TICs Form Luminal Mammary Tumors

We dissociated preneoplastic mammary glands from 5-month-old *MMTV-ErbB2* mice (Guy et al., 1992), an age at which no visible tumors were detected by whole-mount mammary gland analysis (Figure S1A available online). Freshly sorted epithelial subpopulations, including enriched CD24^{med}CD49f^{hi} MaSC (P5), CD24^{med}CD49f^{lo} mature myoepithelial cells (P6) (Shackle-

ton et al., 2006; Stingl et al., 2006), CD24^{hi}CD49f^{lo}CD61⁻ mature luminal cells (P7), and CD24^{hi}CD49f^{lo}CD61⁺ luminal progenitors (P8) (Asselin-Labat et al., 2007), that contained more than 90% TER119⁻/CD31⁻ cells, among which the P5+P6 basal population was 87% positive for cytokeratin 5 (CK5) and the P7+P8 luminal cells were 98% positive for CK8 (Figure 1A; Figures S1B and S1C), were orthotopically transplanted into the #2 mammary fat pads of *Rag1^{-/-}* FVB/N females. Palpable tumors were detected after 2–3 months in 90%–100% of mice transplanted with MaSCs and after 3 months in 60% of mice that received luminal progenitors (Figures 1B and 1C). Surprisingly, myoepithelial cells also formed tumors in 50% and 90% of the recipients after 2 or 3 months, respectively, but nearly no tumors appeared in mice transplanted with mature luminal cells (Figures 1B and 1C). MaSCs and myoepithelial cells did not exhibit a significant difference in tumorigenicity, but tumors derived from MaSCs or myoepithelial cells were significantly larger than those derived from luminal progenitors (Figure 1C). Regardless of their origin, all tumors closely resembled spontaneous tumors from *MMTV-ErbB2* females in histology and were of the luminal type because they expressed the luminal marker cytokeratin 18 (CK18) and not the basal cell marker CK5 (Figure 1D). Thus, even MaSC and mature myoepithelial cells, which express basal cell markers, seem to generate luminal-type tumors. To further characterize the differentiation potential of *MMTV-ErbB2* basal cells and luminal progenitors, we cultured the cells in matrigel. Whereas basal cells formed large spheroids, luminal progenitors formed fewer spheroids and also formed alveolar structures that were not observed in the basal cell cultures (Figures S1D).

IKK α and NIK Promote Basal Cell Expansion and Mammary Tumorigenesis

IKK α is a critical mediator of ErbB2-, but not Wnt- or Ras-induced, mammary tumorigenesis, and its activity is required for TIC self-renewal (Cao et al., 2007). Similar results were obtained using the immortalized ErbB2-induced mammary cancer cell line MT2 (Tan et al., 2011). To identify cellular targets dependent on IKK α , we analyzed preneoplastic mammary glands of 5-month-old *MMTV-ErbB2/Ikka^{AA/+}* and *MMTV-ErbB2/Ikka^{AA/AA}* females by flow cytometry of enriched Ter119⁻CD45⁻CD31⁻ (L⁻) mammary epithelial cells (Figure S1E). IKK α inactivation markedly decreased the CD24^{med}CD49f^{lo}L⁻ basal cell population, whereas there was no significant change in the CD24^{hi}CD49f^{lo}L⁻ total luminal population and a marginal change in CD24^{hi}CD49f^{lo}CD61⁺L⁻ luminal progenitors (Figure S1F). Correspondingly, IKK α inactivation prevented spheroid formation by basal cells but had little effect, if any, on spheroid formation by luminal progenitors, although it did reduce their ability to form alveolar structures (Figure S1D). *MMTV-ErbB2/Ikka^{AA/AA}* mammary glands contained a single layer of CK5⁺ basal cells but were devoid of p63-positive basal cells and nuclear IKK α -positive cells (Figure S1G), suggesting a heterogeneity of the basal cell population. This effect of IKK α inactivation on basal cell number was absent in FVB/N virgins not carrying the *MMTV-ErbB2* transgene (data not shown), explaining why virgin *Ikka^{AA/AA}* females do not exhibit mammary gland defects (Cao et al., 2001). Thus, IKK α is involved in mammary cell proliferation only in response to pregnancy-associated signals, such as RANKL (Asselin-Labat et al., 2010), or upon elevated ErbB2 activity.

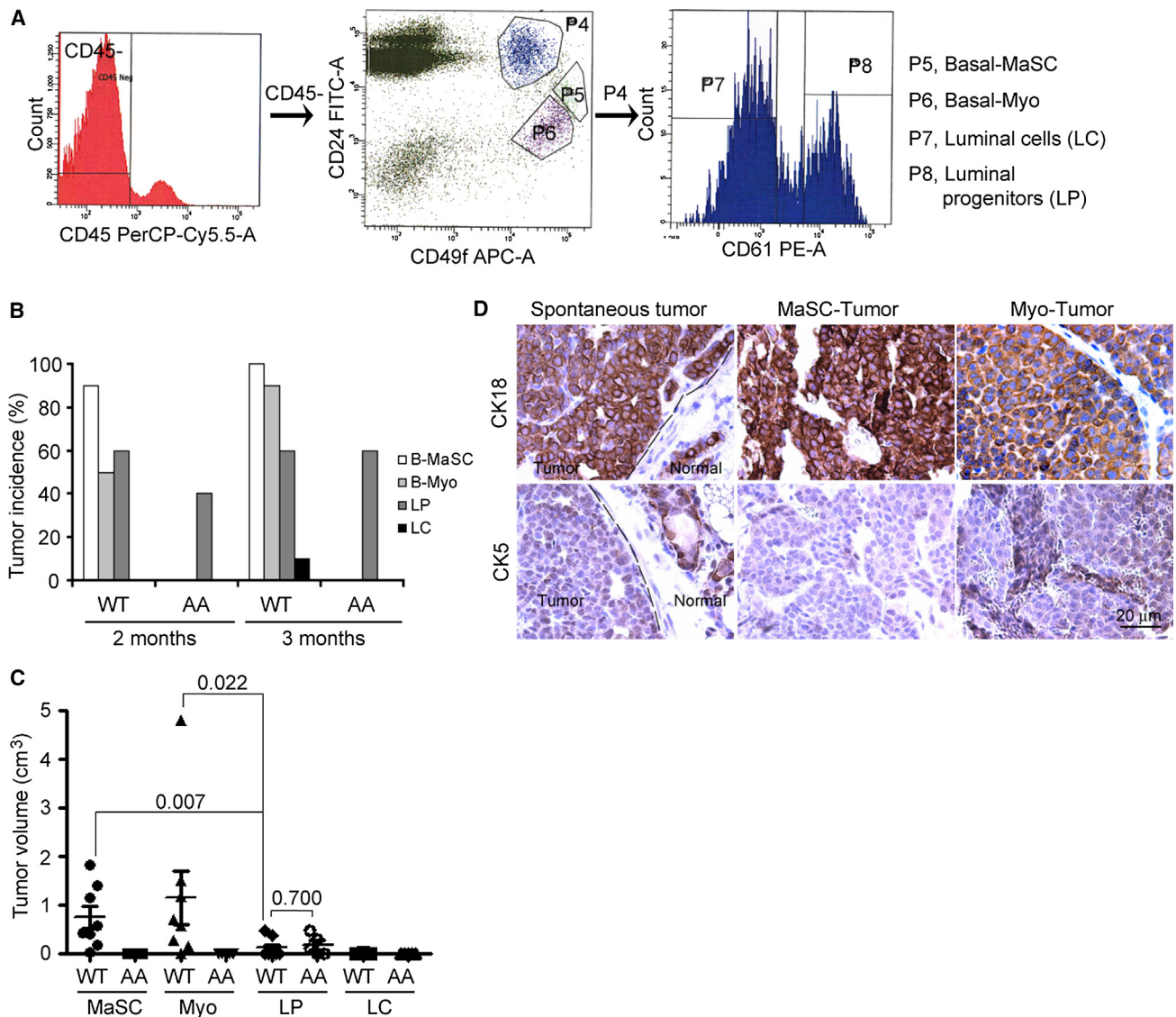


Figure 1. Cellular Origins of ErbB2-Induced Mammary Cancer

(A) Flow cytometry and cell-sorting procedures used to identify the cellular origins of ErbB2-induced mammary cancer.

(B) Mammary tumor incidence 2 and 3 months after orthotopic transplantation of 5×10^4 cells from the indicated sorted populations ($n = 10$ each for *ErbB2*/WT cells; $n = 5$ each for *ErbB2*/*Ikka*^{AA/AA} cells).

(C) Tumor volumes at 3 months after transplantation as in (B).

(D) Immunohistochemical analysis of spontaneous *ErbB2*-induced tumors and tumors generated by transplanted cells. MaSC, mammary stem cells; Myo, mature myoepithelial cells; LP, luminal progenitors; LC, mature luminal epithelial cells; CK5, cytokeratin 5; CK18, cytokeratin 18. See also Figure S1.

We also transplanted purified MaSCs, mature myoepithelial cells, luminal progenitors, and mature luminal cells from *MMTV-ErbB2*/*Ikka*^{AA/AA} females into mammary fat pads of *Rag1*^{-/-} females (Figure 1B). Consistent with a requirement for $\text{IKK}\alpha$ activity in basal cell maintenance, MaSCs or mature myoepithelial cells from *MMTV-ErbB2*/*Ikka*^{AA/AA} mice did not give rise to tumors (Figure 1B). In contrast, *MMTV-ErbB2*/*Ikka*^{AA/AA} luminal progenitors retained tumorigenic potential and gave rise to tumors in 40% and 60% of recipients after 2 or 3 months, respectively (Figures 1B and 1C). These results suggest that $\text{IKK}\alpha$ kinase activity is critical for maintaining basal TICs but is dispensable for expansion of luminal progenitors.

To examine the role of the $\text{IKK}\alpha$ -activating kinase NF- κB -inducing kinase (NIK) (Senftleben et al., 2001), we backcrossed C57B/6 *Nik*^{-/-} mice to the FVB/N background for eight generations and crossed the offspring with FVB/N *MMTV-ErbB2* mice to generate *MMTV-ErbB2*/*Nik*^{+/+}, *MMTV-ErbB2*/*Nik*^{+/-}, or *MMTV-ErbB2*/*Nik*^{-/-} females. *Nik* ablation dramatically reduced the number of preneoplastic mammary gland lesions (Figure 2A) and the number of Ki67-positive mammary epithelial cells (Figures S2A and S2B). *Nik* ablation also reduced the CD24^{med}CD49f⁺L⁻ and CK5⁺ basal cell populations, while having little effect, if any, on the CD24^{hi}CD49f^{lo}L⁻ luminal cell population (Figures 2B and 2C; Figure S2C). *MMTV-ErbB2*/*Nik*^{-/-} females

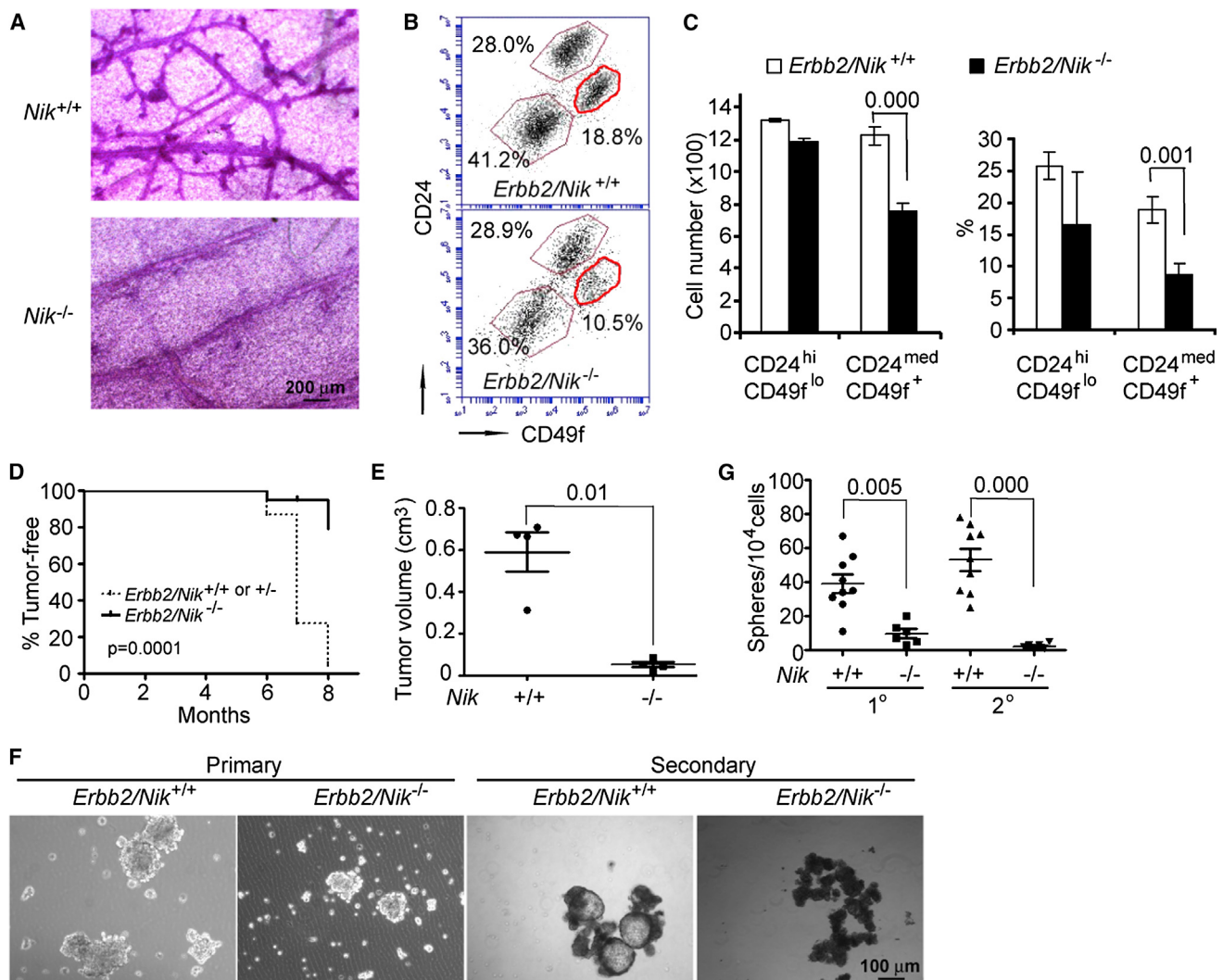


Figure 2. NIK Is Required for ErbB2-Induced Mammary Tumorigenesis and TIC Self-Renewal

(A) Stained whole mounts of preneoplastic mammary glands from 5-month-old *MMTV-ErbB2/Nik^{+/+}* and *MMTV-ErbB2/Nik^{-/-}* females. (B) Flow cytometry of CD45⁻, CD31⁻, and Ter119⁻ (lineage-negative, L⁻) epithelial cells from preneoplastic mammary glands of the indicated mice. (C) Quantitation of cell numbers (left panel) and percentages (right panel) of different mammary epithelial cell populations from (B). Mean $\pm$ SEM (n = 4). (D) Occurrence of mammary tumors in *MMTV-ErbB2/Nik^{+/+}* or *MMTV-ErbB2/Nik^{-/-}* (n = 23) and *MMTV-ErbB2/Nik^{-/-}* (n = 21) females. (E) Volumes of secondary tumors formed by CD45⁻, CD31⁻, Ter119⁻, and CD140a⁻ (L⁻) tumor cells of the indicated *Nik* genotypes. Data represent mean $\pm$ SEM (n = 4). (F) Left panels: primary L⁻ mammary tumor cells from indicated mice were grown as mammospheres. Right panels: secondary mammosphere formation by single cell suspensions derived from primary mammospheres of the indicated genotypes. (G) Quantitation of sphere numbers from (F). Mean $\pm$ SEM (n = 6–9). (C), (D), (E), and (G): exact p values are indicated in the panels. See also Figure S2.

exhibited delayed tumor onset relative to *MMTV-ErbB2/Nik^{+/+}* or *MMTV-ErbB2/Nik^{-/-}* females (Figure 2D). By 8 months of age, only 20% *MMTV-ErbB2/Nik^{-/-}* females developed mammary tumors, whereas 90% of the *MMTV-ErbB2/Nik^{+/+}* and *MMTV-ErbB2/Nik^{-/-}* cohorts exhibited mammary tumors. Notably, *Nik^{-/-}* mice in the FVB/N genetic background developed multi-organ granulocytic infiltration and died between 5 to 9 months (data not shown), thus excluding long-term analysis. To circumvent this complication, we isolated primary epithelial cells from mammary tumors of *MMTV-ErbB2/Nik^{-/-}* and *MMTV-ErbB2/Nik^{+/+}* littermate females and transplanted them into FVB/N

mammary glands. NIK-deficient cancer cells exhibited markedly reduced tumorigenic potential (Figure 2E) and formed smaller and fewer mammospheres relative to NIK-expressing cells and failed to form secondary mammospheres after passage (Figures 2F and 2G). Likewise, NIK-deficient primary tumors gave rise to fewer secondary tumors and almost no tertiary tumors by comparison to NIK-expressing tumors (Figure S2D). We also examined the frequency of TICs in *MMTV-ErbB2/Nik^{+/+}* and *MMTV-ErbB2/Nik^{-/-}* tumors using serial dilution. As few as 100 *MMTV-ErbB2/Nik^{+/+}* primary cancer cells formed tumors in transplanted mice, but it took 10^4 – 10^5 *MMTV-ErbB2/Nik^{-/-}*

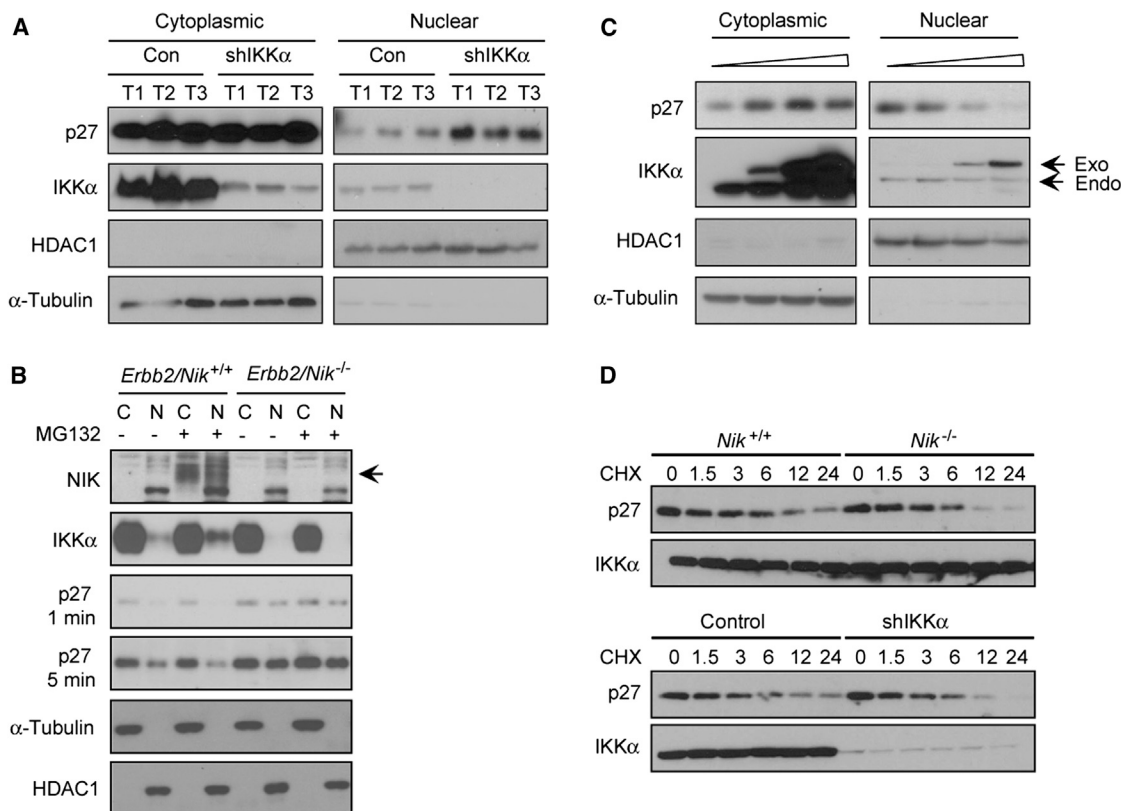


Figure 3. IKK α Controls the Subcellular Localization of p27

(A) IKK α controls nuclear p27 in mammary tumors. Mammary tumors were formed by MT2 cells expressing either control or IKK α shRNAs. Cytoplasmic and nuclear fractions were prepared from three individual tumors of each group and analyzed by immunoblotting with the indicated antibodies.

(B) L⁻ primary mammary tumor cells were isolated from the indicated mice and incubated with MG132, a proteasome inhibitor, for 6 hr. Cytoplasmic and nuclear fractions were prepared, separated by SDS-PAGE, and immunoblotted with the indicated antibodies.

(C) Overexpression of IKK α in MCF7 cells decreases nuclear p27 accumulation. MCF7 cells were transfected with increasing amounts of an IKK α expression vector (0–2 μ g per plate). After 36 hr, nuclear and cytoplasmic fractions were analyzed by immunoblotting.

(D) NIK and IKK α do not affect p27 turnover. L⁻ mammary tumor cells from the indicated mice or MT2 cells expressing control or IKK α shRNA were treated with cycloheximide (CHX) for the indicated times (hr). Gel-separated cell lysates were immunoblotted with the indicated antibodies. See also Figure S3.

cancer cells to form an equivalent number of tumors (Figure S2E). NIK was required for activation and nuclear translocation of IKK α (Figures S2F and S2G).

The NIK-IKK α Module Induces p27 Nuclear Exclusion

IKK α controls expansion of lobuloalveolar cells during pregnancy through its effect on cyclin D1 expression, and an *MMTV-cyclin D1* transgene rescues the lactation defect caused by IKK α inactivation (Cao et al., 2001). However, when we compared cyclin D1 expression in lysates of *MMTV-ErbB2/Ikk α ^{+/+}* or *MMTV-ErbB2/Ikk α ^{AA/AA}* mammary cancer cells, we failed to detect significant differences (Cao et al., 2007). Cyclin D1 and its associated kinase activity, as well as other proteins involved in cell cycle and apoptosis, including p16, p19, E2F1, and Bcl2, were also not significantly altered upon IKK α silencing in MT2 cells (Figures S3A and S3B). Interestingly, the presence of nuclear cyclin D1 was partially correlated with Ki67 nuclear staining in tumors from *MMTV-ErbB2/Ikk α ^{+/+}* female mice, but no significant correlation was between the two was found in *MMTV-ErbB2/Ikk α ^{AA/AA}* tumors or in preneoplastic mammary glands of

either *MMTV-ErbB2/Ikk α ^{+/+}* or *MMTV-ErbB2/Ikk α ^{AA/AA}* females (Figure S3C), suggesting that cyclin D1 may be dispensable for expansion of basal TICs. *Skp2*, whose messenger RNA (mRNA) expression was reported to be controlled by IKK α -mediated NF- κ B activation (Schneider et al., 2006), was also not altered in tumors from IKK α -silenced cells (Figure S3D). However, the total amount of p27 was occasionally elevated in some of the tumors derived from IKK α -silenced cells. Although this effect was variable, further analysis revealed that the amount of nuclear p27, a negative regulator of G1-S cell-cycle transition and ErbB2-induced mammary tumorigenesis (Muraoka et al., 2002), was consistently elevated in IKK α -silenced MT2 cells, whereas cytoplasmic p27 was barely affected (Figure 3A). We also purified L⁻ primary cancer cells from *ErbB2/Nik^{+/+}* and *ErbB2/Nik^{-/-}* tumors and treated them with the proteasome inhibitor MG-132 to prevent constitutive NIK turnover. This resulted in NIK accumulation and enhanced IKK α nuclear translocation in *MMTV-ErbB2/Nik^{+/+}* cells, as well as reduced nuclear p27 (Figure 3B). p27 levels were higher in *MMTV-ErbB2/Nik^{-/-}* cells regardless of MG-132 treatment. We also found that

spontaneous ErbB2-induced tumors contained many cells with nuclear IKK α , and in most of these cells, p27 was excluded from the nucleus (Figure S3E). These results suggest that the NIK-IKK α module is a negative regulator of nuclear p27 accumulation. Reduced nuclear p27 was also found in MCF7 human breast cancer cells that were transiently transfected with an IKK α expression vector; increased IKK α expression resulted in a dose-dependent decrease in nuclear p27 and increased cytoplasmic p27 (Figure 3C). Silencing of IKK α expression in MCF7 or T47D cells led to nuclear accumulation of p27, without significantly influencing IKK β expression and nuclear RelA/p65 (Figure S3F). Notably, neither NIK nor IKK α had an effect on the turnover of p27 (Figure 3D).

IKK α Phosphorylates p27 and Promotes Its Nuclear Exclusion

To examine if p27 is a substrate for IKK α , we carried out an *in vitro* kinase assay. IKK α purified from baculovirus-infected insect cells phosphorylated p27 better than I κ B α , the original IKK α substrate (Figure 4A). Endogenous IKK α from RANKL-stimulated mammary cancer cells, whose activation was NIK-dependent, also phosphorylated p27 (Figure 4B). Only the nuclear form of IKK α from RANKL-stimulated cells phosphorylated p27, whereas the cytoplasmic form was inactive (Figure 4C). IKK α kinase activity was required for p27 phosphorylation as only immunoprecipitated constitutively active IKK α (EE), in which activation loop serines were replaced with glutamates, phosphorylated p27, whereas kinase-dead IKK α (K/A) or inactive IKK α (AA) mutants failed to do so (Figure 4D).

To determine if endogenous p27 is phosphorylated by IKK α , MT2 cells transduced with control or IKK α small hairpin RNAs (shRNAs) were labeled with ³²P-orthophosphate in the presence or absence of the nuclear export inhibitor leptomycin B (LMB). Neither IKK α expression nor LMB treatment affected phosphorylation of cytoplasmic p27, but LMB treatment enhanced nuclear p27 phosphorylation in control cells, but not in IKK α -silenced cells (Figures 4E and S4A). Mass spectrometric analysis of p27 that was phosphorylated *in vitro* by IKK α indicated that the main phosphoacceptors were four serines (S) and one threonine (T): S12, T42, S175, S178, and S183 (Figure S4B). The sequences around these phosphoacceptor sites exhibit similarity to known and consensus IKK phosphorylation sites (Figure S4C) and are conserved across mammalian species (Figure S4D). Alignment of p21/Cip1 and p57/Kip2 with the p27 sequence revealed that T42 and S183 are also conserved in p57/Kip2, but not in p21 (data not shown). Replacement of S175 and S183 with alanines reduced the extent of IKK α -mediated p27 phosphorylation (Figure S4E). Wild-type (WT) p27 and the S175A and S183A mutants were *in vitro* phosphorylated by IKK α and subjected to two-dimensional tryptic phosphopeptide mapping. Replacement of either S175 or S183, which are located within a single tryptic peptide (Figure S4B, blue color), with alanines reduced the phosphorylation of the corresponding peptide (Figure S4F). We also detected p27 phosphorylation at S175 and S183 using phospho-S175- or phospho-S183-specific antibodies. Interestingly, whereas the S183A mutation had no effect on S175 phosphorylation, the S175A substitution affected S183 phosphorylation by recombinant IKK α , suggesting that p27 may be sequentially phosphorylated, at least *in vitro* (Figure 4F). How-

ever, in MDA-MB-231 breast cancer cells, IKK α silencing reduced S183 phosphorylation, while having no significant effect on S175 phosphorylation (Figure S4G). Although phosphorylation of endogenous p27 at S183 was barely detectable, IKK α silencing caused increased nuclear accumulation of endogenous p27 and a significant reduction in both cytoplasmic and nuclear S183 phosphorylation of transiently expressed exogenous p27 in ErbB2-positive SKBR3 cells (Figure 4G). Overexpression of activated IKK α (EE), but not inactive IKK α (AA), induced S183 phosphorylation in breast cancer cells that was abolished by the S183A mutation, but not by the S175A mutation (Figure 4H). Consistent with a role for IKK α in regulating p27 subcellular localization, silencing of IKK α in MDA-MB-231 or MT2 cells reduced CDK2 kinase activity measured with p27 as a substrate (Figure S4H) and overexpression of IKK α (EE), but not IKK α (AA), increased CDK2 kinase activity (Figure S4I). We examined the effect of the two mutations on the nuclear localization of p27 and found that the S183A substitution enhanced p27 nuclear localization in human BCa cells, whereas a phosphomimetic S183E substitution interfered with nuclear localization (Figures 4I and S4J–S4L). Ectopic expression of different p27 variants had no effect on the subcellular localization of endogenous p27 (Figures 4I and S4L). Consistent with these observations, IKK α silencing resulted in decreased cell proliferation as indicated by fewer cells at the S/G2/M phases (Figure S4M) or fewer BrdU-positive cells in MT2-generated tumors (Figure S4N). These data suggest that IKK α -mediated phosphorylation promotes the nuclear export of p27, resulting in enhanced CDK2 activity.

Reduced p27 Expression Restores Basal Cell Expansion and Tumorigenesis in *Ikka*^{AA/AA} Mice

Kip1 is a haploinsufficient suppressor of ErbB2-induced mammary tumorigenesis (Muraoka et al., 2002). To evaluate the effect of reduced *Kip1* dosage on ErbB2-stimulated basal cell expansion, we crossed *MMTV-ErbB2* mice with *Kip1*^{+/-} mice. The preneoplastic mammary glands of 4-month-old *MMTV-ErbB2/Kip1*^{+/-} mice contained more pronounced side and terminal buds than those of *MMTV-ErbB2/Kip1*^{+/+} mice, but there were hardly any differences in appearance of *Kip1*^{+/-} and *Kip1*^{+/+} mammary glands without the *MMTV-ErbB2* transgene (Figure S5A). Flow cytometry revealed more CD24^{med}CD49f⁺L⁻ basal cells in mammary glands of *MMTV-ErbB2/Kip1*^{+/-} mice (Figures S5B and S5C). To place p27 as a downstream effector of the NIK-IKK α module, we generated *MMTV-ErbB2/Ikka*^{AA/+}/*Kip1*^{+/+}, *MMTV-ErbB2/Ikka*^{AA/+}/*Kip1*^{+/-}, *MMTV-ErbB2/Ikka*^{AA/AA}/*Kip1*^{+/+}, and *MMTV-ErbB2/Ikka*^{AA/AA}/*Kip1*^{+/-} mice. As described above, reduced *Kip1* dosage enhanced mammary ductal branching and expansion (Figure 5A). The mammary glands of 5-month-old *MMTV-ErbB2/Ikka*^{AA/AA} females were nearly devoid of side and terminal buds relative to *MMTV-ErbB2/Ikka*^{AA/+} females, but this defect was reversed upon deletion of one *Kip1* allele (Figure 5A). IKK α inactivation reduced the number of CD24^{med}CD49f⁺L⁻ (Figures 5B and 5C) and CK5-positive (Figures 5D and 5E) basal cells in *MMTV-ErbB2* mammary glands without significantly affecting the number of CD24^{hi}CD49f^{lo}L⁻ (Figures 5B and 5C) and CK18-positive luminal cells (Figures 5D and 5E). All of these defects were reversed by monoallelic *Kip1* deletion (Figures 5B–5D and 5E). These data suggest that IKK α and p27 function in the same pathway to regulate basal

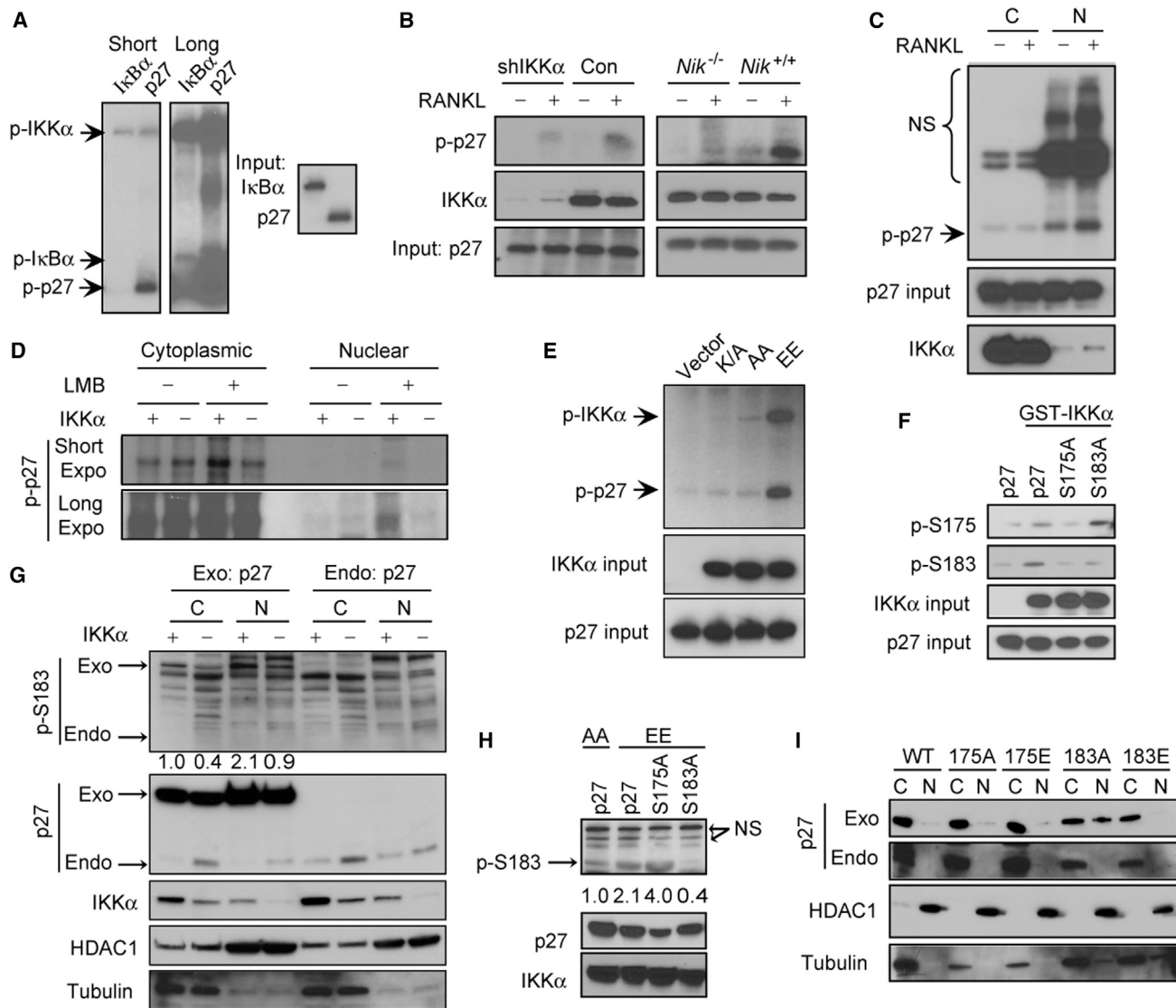


Figure 4. p27 Is a Direct Substrate for Nuclear IKK α

(A) In vitro kinase assays using GST-IKK α purified from insect cells and HIS-p27 or HIS-IκB α purified from *Escherichia coli*.

(B) In vitro kinase assays using endogenous IKK α immunoprecipitated from control or IKK α -silenced MT2 cells or primary mammary tumor cells of the indicated genotypes incubated with or without RANKL for 2 hr.

(C) Nuclear IKK α is a potent p27 kinase. MT2 cells were treated with or without RANKL. After 2 hr, cytoplasmic and nuclear lysates were immunoprecipitated with an IKK α antibody. The immune complexes were incubated with HIS-p27, and kinase reactions were conducted.

(D) HEK293T cells were transiently transfected with vectors encoding HA-tagged kinase dead (K/A), inactive (AA), or activated (EE) IKK α . Thirty-six hours later, lysates were prepared and immunoprecipitated with HA antibody. Immune complex kinase assays with HIS-p27 were performed as above.

(E) ³²P metabolic labeling of MT2 cells expressing control or IKK α shRNA with or without leptomycin B (LMB) treatment. After 4 hr, endogenous p27 was immunoprecipitated, separated by SDS-PAGE, and autoradiographed.

(F) Kinase assays using purified GST-IKK α and HIS-p27 or the indicated p27 mutants. Kinase reactions were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

(G) SKBR3 cells expressing control (+) or IKK α (-) shRNAs were transiently transfected with YFP-p27 (exo) or left untransfected (endo) for 16 hr. Cytoplasmic and nuclear lysates were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

(H) SKBR3 cells were transiently transfected with vectors encoding the indicated p27 variants, together with IKK α (AA) or IKK α (EE) expression vectors. After 36 hr, cell lysates were gel analyzed by immunoblotting.

(I) S183 phosphorylation regulates p27 nuclear accumulation. SKBR3 cells were transiently transfected with the indicated p27 variants. After 36 hr, cytoplasmic and nuclear lysates were prepared and analyzed by immunoblotting. See also Figure S4.

cell expansion. Inactivation of IKK α also inhibited the proliferation of mammary epithelial cells assessed by Ki67 staining (Figures S5E and S5F). This defect was also reversed by ablation

of one *Kip1* allele (Figures S5E and S5F). We examined the self-renewal ability of L⁻ TICs isolated from tumors of the above four genotypes using the mammosphere formation assay.

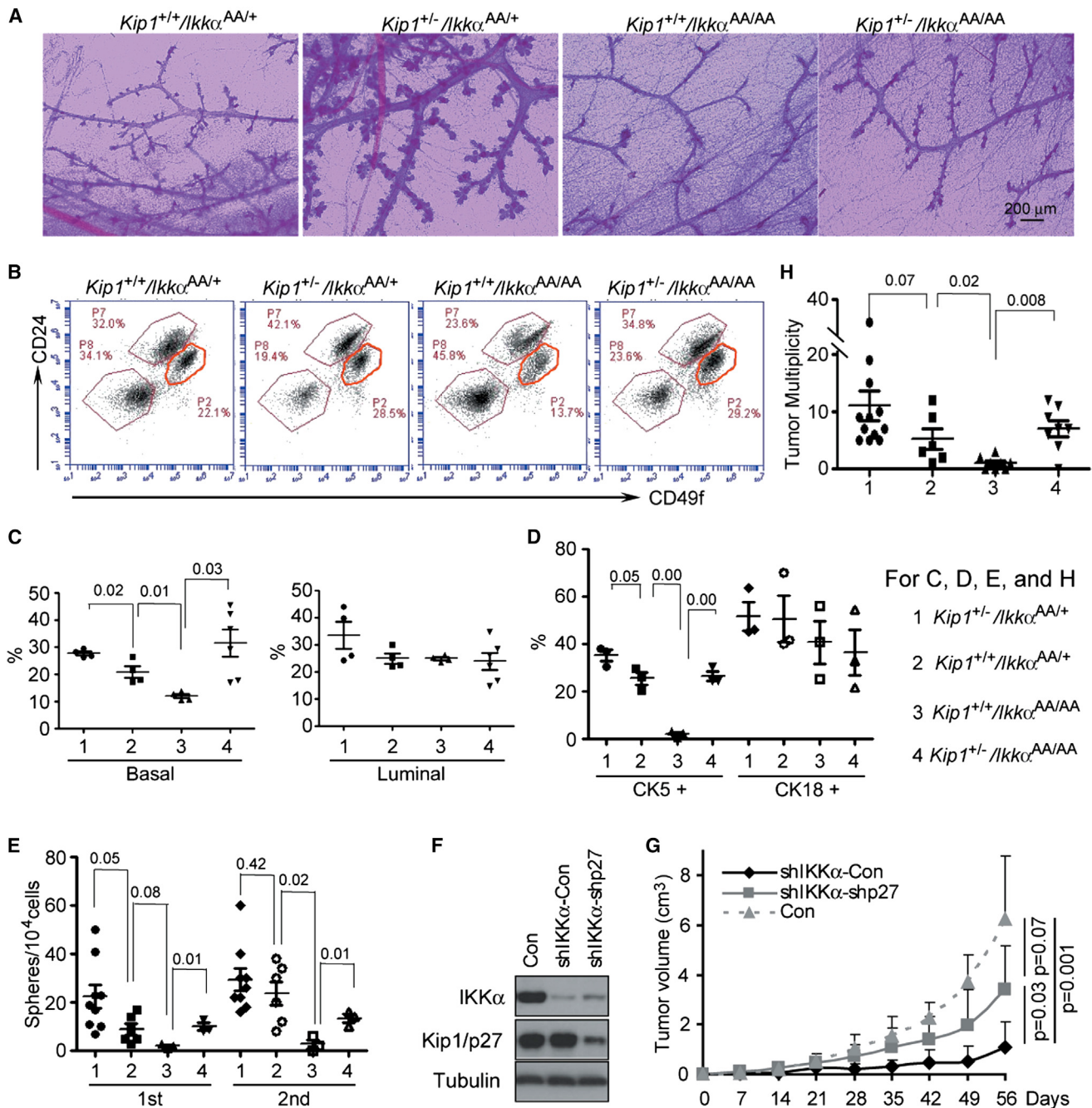


Figure 5. Reduced p27 Expression Rescues Proliferation and Tumorigenic Defects Caused by IKK α Inactivation

(A) Preneoplastic mammary glands of 5-month-old *MMTV-ErbB2* females of the indicated genotypes were subjected to whole-mount analysis.
 (B) Flow cytometric analysis of L⁺ mammary epithelial cells from preneoplastic mammary glands of 5-month-old females of the indicated genotypes.
 (C) Quantitation of the different cell populations analyzed in (B).
 (D) Quantitation of CK5- and CK18-positive L⁺ mammary epithelial cells in preneoplastic mammary glands from 5-month-old females of the indicated genotypes. Mean $\pm$ SEM (n = 3).
 (E) Quantitation of mammosphere numbers formed by L⁺ mammary cancer cells of the indicated genotypes or by trypsinized primary mammospheres. Mean $\pm$ SEM (n = 4–6).
 (F) MT2 cells infected with lentiviruses expressing control, IKK α , or IKK α +p27 shRNAs were analyzed for IKK α and p27 expression by immunoblotting.
 (G) Mammary tumor formation by orthotopically transplanted MT2 cells from (F). Tumor volume = length $\times$ width² $\times$ 0.52. Values represent mean $\pm$ SEM (n = 5).
 (H) Quantitation of tumor multiplicity in 7- to 8-month-old females of the indicated genotypes. Tumor multiplicity was determined by counting all visible tumors in each mouse. Mean $\pm$ SEM (n = 6–12). (C)–(E), (G), and (H): exact p values are indicated. See also Figure S5.

MMTV-ErbB2/Ikk $\alpha^{AA/AA}$ cells formed small primary spheres that could not be successfully propagated upon in vitro passage (Figures 5E and 5G). *Kip1* heterozygosity rescued this defect and led to efficient formation of both primary and secondary mammospheres by *MMTV-ErbB2/Ikk $\alpha^{AA/AA}/Kip1^{+/-}$* TICs. Silencing of p27 expression restored tumorigenic growth in IKK α -silenced MT2 cells (Figures 5F and 5G). Most importantly, *MMTV-ErbB2/Ikk $\alpha^{AA/AA}$* females exhibited reduced tumor multiplicity relative to *MMTV-ErbB2/Ikk $\alpha^{AA/+}$* females, which was completely rescued by ablating one *Kip1* allele (Figure 5H).

Nuclear IKK α Correlates with Reduced Nuclear p27 and Increased Metastasis in BCa

Analysis of the expression array data set GSE2603, which is based on 121 human BCa specimens, including 81 with full prognostic records (Minn et al., 2005), revealed a significant positive correlation between IKK α mRNA amounts and tumor size (Figure 6A). IKK α mRNA abundance also exhibited a positive correlation with the incidence of metastasis (Figure 6B) and a negative correlation with metastasis-free survival (Figures 6C–6E). Curiously, expression of TRAF2 and TRAF3, negative regulators of NIK-IKK α signaling (Vallabhapurapu et al., 2008), exhibited inverse correlation with metastatic events (Figure 6B). In agreement with IKK α 's role in control of mouse mammary epithelial cell proliferation, gene set enrichment analysis (GSEA), a computational method that determines whether a defined set of genes shows statistically significant and concordant differences between two biological states (Subramanian et al., 2005), revealed that 16 gene sets related to cell-cycle progression were significantly upregulated in tumors with high IKK α mRNA content (Figure S6A). Other pathways that were upregulated in the high IKK α group included genes involved in proteasome-mediated proteolysis, DNA replication and synthesis, RNA processing, and mRNA translation (Figure S6A), all of which are critical for cell-cycle progression. A significant enrichment of mRNAs encoding cell-cycle regulators, including CCNB1, CCNB2, CCND3, CCNE2, CCNA2, CDK2, and CDK7, was also observed in the high IKK α group (Figure S6B). Based on IKK α mRNA expression, the above cell-cycle regulators formed three distinct clusters: 51% of the cancers expressed the lowest amounts of cell-cycle regulators clustered in the low IKK α group, and 32% of BCa expressed intermediate amounts of cell-cycle regulators and clustered in the medium IKK α group, whereas 21% expressed the highest amounts of cell-cycle regulators and were in the high IKK α group (Figure S6B).

We stained an array of human BCa specimens with antibodies to IKK α and p27 (Figure S6C). The majority of invasive ductal carcinomas (IDCs) showed a mutually exclusive relationship between IKK α and p27 such that 25.6% (10/39) of IDCs without metastasis and 73.7% (28/38) of IDCs with metastasis exhibited high nuclear IKK α and low nuclear p27 (Figures 6F and 6G; Figure S6C). Among IDCs with valid ER, PR, and ERBB2 status, 45% ER/PR⁺ (18/40), 84% ERBB2⁺ (14/17), and 62% triple-negative (11/17) IDCs exhibited high nuclear IKK α and low nuclear p27. We confirmed that RANK, a potential upstream activator of NIK/IKK α during mammary basal cell expansion (Asselin-Labat et al., 2010) and tumorigenesis (Schramek et al., 2010), is expressed in 22% of IDCs (16/72), among which 87.5% (14/16) exhibit nuclear IKK α expression (Figure S6D).

Our analysis plus the known correlation between p27 subcellular distribution and breast cancer prognosis (Shin et al., 2002; Viglietto et al., 2002) underscores the importance of this pathway in initiation and progression of human BCa and suggests that the role of IKK α in p27 regulation may be conserved across different mammalian species.

In summary, we identified different TICs for ErbB2-induced mammary tumorigenesis. The NIK/IKK α module is critical for basal TIC expansion during tumor initiation primarily by phosphorylating p27 and driving its nuclear export (Figure 6H). However, basal TICs-formed tumors have a final luminal appearance, suggesting a conversion from basal to luminal lineage. Indeed, we observed that purified basal cells cultured in matrigel formed three major types of spheroid structures: (1) hollow spheres with one or two layers of cells (data not shown), (2) hollow spheres with multiple cell layers at some regions that contained cells that were double positive for CK5 and CK8 (Figure S6E), suggesting a transition from basal TICs to early progenitors (Liu et al., 2008; Wang et al., 2008), and (3) solid spheres in which most cells were CK8-positive with hardly any CK5 expression (Figure S6F), mimicking the phenotype of basal TIC-derived tumors (Figure 1).

DISCUSSION

Early observations that ErbB2-induced mammary cancer is of the luminal type (Guy et al., 1992) led to the suggestion that ErbB2-induced tumors are derived from luminal progenitors (Visvader, 2011). A previous study also revealed that unlike the *Wnt* oncogene, the *ErbB2* oncogene does not cause basal cell expansion in preneoplastic mammary glands (Shackleton et al., 2006), suggesting that ErbB2-induced tumors may originate from luminal progenitors. Our study, however, suggests that ErbB2-induced mammary cancer can arise from both basal cells and luminal progenitors. Although our current purification procedures do not yield 100% pure populations, one can exclude the possibility that the tumors formed by transplanted basal cells originate from contaminating luminal cells. It is quite unlikely that the ~10% luminal cell contamination in the basal cell population (Figure S1C) can give rise to tumors that are larger and more numerous than those formed by 90% pure luminal progenitors. Furthermore, inactivation of IKK α had no obvious effect on the luminal population, both in preneoplastic mammary glands and in tumors, but it had a clear effect on cells that express basal cell markers and on the tumorigenic potential and mammosphere-forming ability of isolated basal cells. These results suggest that a small portion of the basal cell population may serve as the TICs for ErbB2-induced tumors and that the expansion of such cells that are capable of transdifferentiation into luminal cells is IKK α -dependent. These findings are supported by previous identification of PI-MEC in parous and nulliparous mammary glands (Wagner et al., 2002). These cells express CD49f (Matulka et al., 2007), the same basal cell marker used to identify multipotent MaSC (Shackleton et al., 2006), and were proposed to be the tumorigenic target for ErbB2 (Henry et al., 2004). However, another study has pointed out a different PI-MEC population present within the CD24^{hi}CD49f^{lo} luminal cell fraction as an alternative target for ErbB2 (Jeselsohn et al., 2010). Our results suggest that ErbB2 can target both basal cells and

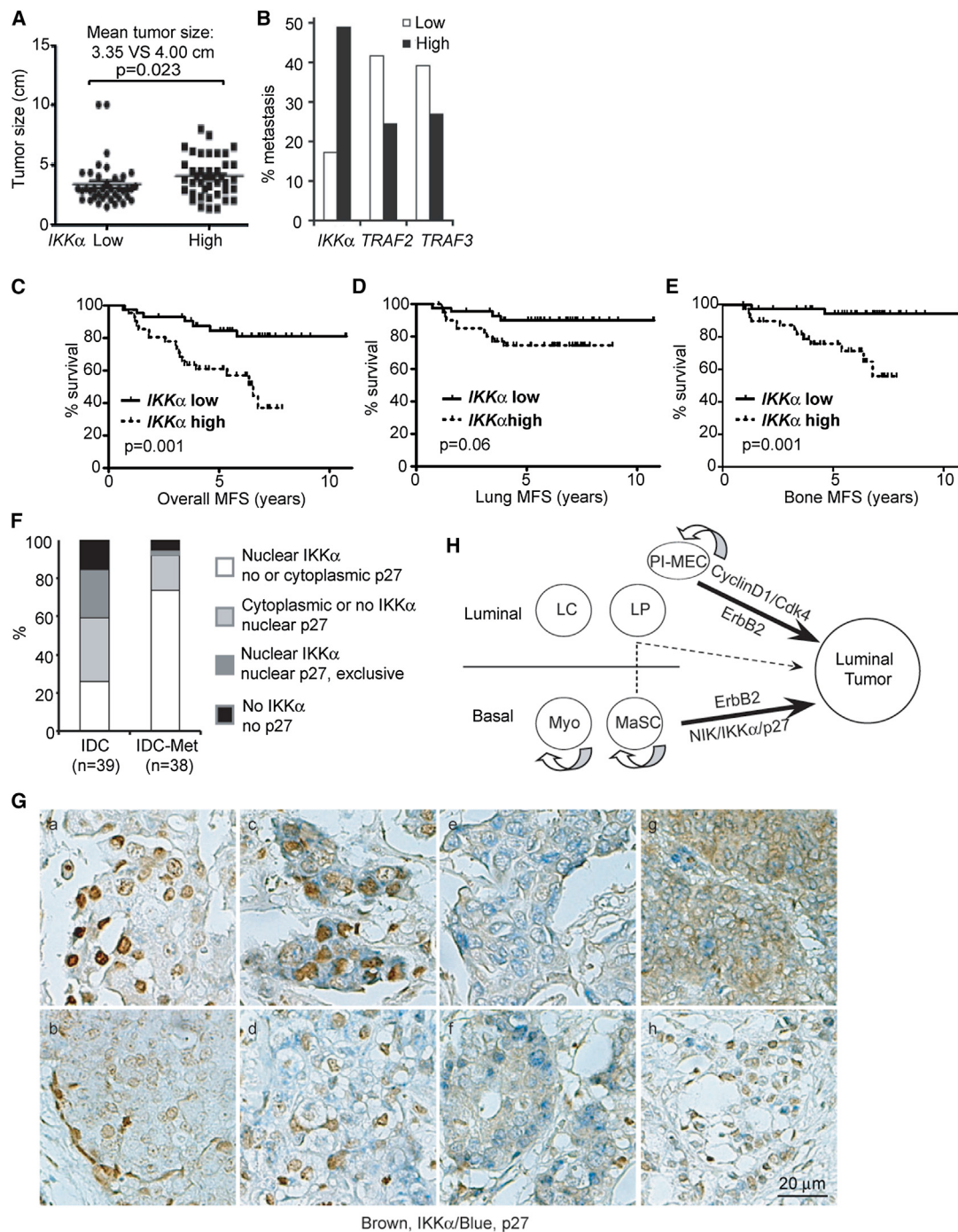


Figure 6. IKK α Expression and Nuclear Presence Correlate with Tumor Size, Metastasis, and p27 Distribution in BCa

(A–E) The Affymetrix data set GSE2603, which includes xenografted BCa cells and 81 BCa specimens, was downloaded and analyzed. Clinical breast cancer specimens were divided into two groups based on mRNA expression. (A) Correlation of *IKK α* mRNA with primary tumor size. (B) Percentage of metastatic tumors in the high and low *IKK α* , *TRAF2*, and *TRAF3* groups. (C–E) Correlation between *IKK α* mRNA and overall metastasis-free survival (C), lung metastasis-free survival (D), and bone metastasis-free survival (E).

(F and G) The subcellular distribution of *IKK α* and p27 in human invasive ductal carcinomas ($n = 39$ IDCs without metastasis and $n = 38$ IDCs with metastasis) was determined by immunohistochemistry staining of a BCa tissue array. (a) and (b) represent specimens with nuclear *IKK α* /no p27; (c) and (d) represent specimens with nuclear *IKK α* /cytoplasmic p27; (e) and (f) represent specimens with *IKK α* -negative/nuclear p27; (g) represents specimens with cytoplasmic *IKK α* /nuclear p27; and (h) represents specimens with nuclear *IKK α* /nuclear p27. Brown, *IKK α* ; blue, p27.

(H) Schematic summary of cellular origins of ErbB2-induced mammary tumors. NIK-IKK α -p27 signaling targets basal cells to promote ErbB2-induced tumorigenesis. It is unknown if differentiation of basal cells into luminal progenitors is required for luminal tumor formation (dotted line and arrow). Cyclin D1, on the other hand, targets a subpopulation of PI-MECs. See also Figure S6.

luminal progenitors in nulliparous mammary glands. In addition to ErbB2, Notch activation (Bouras et al., 2008) or loss of BRCA1 (Molyneux et al., 2010) can target both basal and luminal cells. The final luminal appearance of all ErbB2-induced tumors may be explained by the commitment of ErbB2-transformed basal cells to luminal differentiation. Furthermore, IKK α -dependent and ErbB2-transformed basal cells may become luminal progenitors (Figure 6H, dotted arrow) that are distinct from luminal PI-MECs, the proposed target of cyclin-D1-associated CDK activity (Jeselson et al., 2010). Conversion of MaSC to luminal progenitors was also seen upon Notch activation (Bouras et al., 2008). Conversely, basal mammary tumors initiated by BRCA1 loss are derived from a subset of luminal progenitors (Lim et al., 2009; Molyneux et al., 2010), suggesting that different oncogenic events can cause TIC transdifferentiation. Indeed, we observed that purified basal cells cultured in matrigel formed hollow spheres with multiple cell layers at some regions in which double CK5- and CK8-positive cells were identified (Figure S6E), suggesting a transition from basal TICs to early progenitors (Liu et al., 2008; Wang et al., 2008).

The NIK-IKK α module is only required for maintenance of CD24^{med}CD49f⁺L⁻ basal cells in ErbB2-induced mammary tumors and is dispensable for maintenance of luminal progenitors. Given that IKK α activity is needed for ErbB2-induced mammary tumorigenesis and for the propagation of such tumors upon transplantation (Cao et al., 2007), our results suggest that IKK α -dependent basal cells are the major TIC type in ErbB2-induced mammary cancer. This connection between ErbB2 and IKK α does not seem to occur during normal development, because ErbB2 is important for virgin ductal morphogenesis (Jackson-Fisher et al., 2004), but IKK α is only needed for pregnancy-induced lobuloalveolar expansion (Cao et al., 2001). Furthermore, whereas IKK α controls lobuloalveolar expansion through cyclin D1 (Cao et al., 2001), the NIK-IKK α module does not regulate cyclin D1 expression during ErbB2-induced tumorigenesis. Instead, the NIK-IKK α cascade acts, at least in part, by decreasing the amount of nuclear p27. The role of p27 in normal mammary gland development is controversial (Davison et al., 2003; Muraoka et al., 2001), but it is a well-established haploinsufficient suppressor of ErbB2-induced mammary cancer (Muraoka et al., 2002). We found that IKK α phosphorylates p27 at S183 to cause its nuclear export. In human BCa, an inverse correlation between nuclear IKK α and nuclear p27 was found in about 73.7% of metastatic IDCs but only in 25% of non-metastatic IDCs, suggesting that the prometastatic activity of IKK α (Tan et al., 2011) correlates with its ability to control p27 distribution and function. It remains to be seen, however, whether the human TICs, within which IKK α induces nuclear exclusion of p27, are of basal origin. Phosphorylation-induced p27 nuclear export is a common mechanism in human cancers that relieves the inhibitory effect of p27 on cyclin:CDK2 complexes (Shin et al., 2002; Viglietto et al., 2002). p27 is phosphorylated at several different sites by distinct kinases. S10 phosphorylation was linked to ERK1/2 (Foster et al., 2003), CDK5 (Kawauchi et al., 2006), or nuclear kinase-interacting stathmin (KIS) (Boehm et al., 2002), and it controls the nuclear export or stability of p27. AKT phosphorylates p27 at T157, which is located within its nuclear localization signal (NLS), and causes its cytoplasmic retention in advanced human BCa (Shin et al., 2002; Viglietto et al.,

2002). Other oncogenic kinases also phosphorylate p27 and regulate its subcellular localization and/or turnover (Lu and Hunter, 2010). We now add the NIK-IKK α module as another important regulator of p27 subcellular localization. At this stage, our results suggest that IKK α -mediated p27 phosphorylation has no effect on p27 turnover. However, we did observe that some human BCa specimens with positive nuclear IKK α exhibit reduced or no p27 expression.

IKK α is involved in several signaling pathways that regulate tumor initiation, progression, or metastasis. Besides its role in suppression of maspin, an inhibitor of metastasis in prostate (Luo et al., 2007) and breast (Tan et al., 2011) cancers, IKK α was also reported to regulate cancer cell invasion through classical NF- κ B activation (Merkhofer et al., 2010). In hepatocellular carcinoma, IKK α activates Notch signaling by phosphorylating FOXA2 (Liu et al., 2012). Notch activation is critical for commitment of MaSC to luminal differentiation and leads to mammary tumorigenesis (Bouras et al., 2008). Although Notch activation cannot explain the role of IKK α in expansion of basal TICs, it will be interesting to determine if Notch is activated by IKK α -mediated phosphorylation of Foxa2 during conversion of basal TICs into luminal progenitors.

The upstream signal responsible for activation of the NIK-IKK α module during ErbB2-induced tumorigenesis remains elusive. In human IDCs, IKK α nuclear staining is not tightly linked to the ER, PR, or ERBB2 status, suggesting an ERBB2-independent mechanism of IKK α activation. It is reasonable to speculate that RANK signaling may be responsible for basal cell expansion in ErbB2-induced tumors, as it does in normal mammary glands stimulated with progesterone (Asselin-Labat et al., 2010; Joshi et al., 2010). Indeed, a recent study found that elevated RANK expression is linked to metastasis in human BCa (Palafox et al., 2012). However, RANKL, which is required for RANK activation, is produced either by progesterone-stimulated normal luminal epithelial cells or tumor-infiltrating FoxP3⁺ T cells in advanced ErbB2-induced tumors, and it is not present during early stages of ErbB2 tumorigenesis (Gonzalez-Suarez et al., 2010; Tan et al., 2011). If RANK signaling is responsible for IKK α activation in ErbB2-induced cancer, it should take place during a narrow window when progesterone increases at diestrus, resulting in expansion of CD24^{med}CD49f⁺L⁻ basal cells (Joshi et al., 2010). However, other signals may maintain NIK-dependent IKK α activation once progesterone-induced RANKL expression had subsided. Perhaps, the upregulation of RANK results in its synergistic interaction with other signaling molecules, thereby generating a signal that leads to NIK stabilization and IKK α activation. However, in those human IDCs with nuclear IKK α staining that are RANK negative, other signaling pathways are likely to activate the NIK-IKK α module.

EXPERIMENTAL PROCEDURES

Mice and Human BCa Tissue Array

Mice were maintained under specific pathogen-free conditions, and experimental protocols were approved by the University of California, San Diego Animal Care Program, following National Institutes of Health (NIH) guidelines. *MMTV-ErbB2*-tg and *Nik*^{-/-} (Yin et al., 2001) mice were intercrossed for eight generations to generate mice of the expected genotypes in the FVB/N background. *MMTV-ErbB2/Ikk*^{AA/AA} male mice were further crossed with *Kip1*^{+/-} females (Kiyokawa et al., 1996) of the C57BL/6 background. Preneoplastic

mammary glands were harvested at 5 months of age unless otherwise indicated. Tumors were considered established when they became palpable for two consecutive weeks. One million cancer cells were injected into the #2 mammary fat pad for orthotopic transplantation unless otherwise indicated. Tumor size was measured using a caliper and volume was calculated as length $\times$ width² $\times$ 0.52.

Human BCa tissue array was kindly provided by Dr. Jin Q. Cheng at the H. Lee Moffitt Cancer Center. All primary BCa specimens were obtained from patients who underwent surgery at the H. Lee Moffitt Cancer Center, and samples were deidentified prior to our analysis. The Affymetrix data set GSE2603 was previously published, and all samples were deidentified prior to our analysis (Minn et al., 2005).

Harvesting Mammary Epithelial Cells and Flow Cytometry

Single cell suspensions of preneoplastic mammary glands or tumors were prepared (Shackleton et al., 2006) with slight modification. Briefly, tissues were harvested and cut into small pieces, followed with 300 U/ml collagenase and 100 U/ml hyaluronidase (Stemcell, Vancouver, BC, Canada) digestion for 6 hr in 2% FCS-containing HBSS. Organoids were sequentially resuspended in 0.25% trypsin-EDTA (Mediatech, Corning, NY, USA) for 3 min, 5 μ g/ml dispase I (Stemcell), and 0.1 μ g/ml DNase (Worthington, Lakewood, NJ, USA) for 1 min before filtration through a 40 μ m mesh and antibody staining. Mammary or tumor epithelial cells were magnetically purified using a Mammary Epithelial Cell Enrichment Kit (Stemcell) to remove lineage-positive (CD45-, CD31-, and Ter119-positive) cells, followed by surface-labeling with CD24, Cd49f, and CD61 antibodies and 7-AAD for cell viability before flow cytometric analysis.

Statistics

All results wherever necessary were subjected to statistical analyses. A two-tailed Mann-Whitney test, nonparametric without assuming Gaussian distribution, was performed for most studies. A Kaplan-Meier curve (for tumorigenesis study and metastasis-free survival of human patients) was generated by Prism software and analyzed with a log rank (Mantel-Cox) test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.ccr.2013.03.012>.

ACKNOWLEDGMENTS

We thank Santa Cruz Biotechnology, Cell Signaling, and GeneTex for providing antibodies, T. Hunter and J. Meisenhelder for discussions and technical support, J. Cheng for providing human BCa tissue array, S. Dowdy and C. Denicourt for sharing reagents, and A. Koff for sharing *Kip1*^{+/-} mice. W.Z. was supported by a postdoctoral fellowship from Susan G. Komen for the Cure (KG080649) and a K99/R00 award from the National Institutes of Health (NIH) (CA158055). X.W. was supported by a postdoctoral fellowship from Susan G. Komen for the Cure (KG111506). A.S. was supported by an NIH training grant (T32CA121938). Work was also supported by an NIH grant (CA127923) to M.K., who is an American Cancer Society Research Professor.

Received: June 23, 2012

Revised: October 30, 2012

Accepted: March 15, 2013

Published: April 18, 2013

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