JCI The Journal of Clinical Investigation

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J Clin Invest. 2011;121(3):866-879. https://doi.org/10.1172/JCI42928.

Research Article Oncology

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Hunk is required for HER2/neu-induced mammary tumorigenesis

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Understanding the molecular pathways that contribute to the aggressive behavior of human cancers is a critical research priority. The SNF1/AMPK-related protein kinase Hunk is overexpressed in aggressive subsets of human breast, ovarian, and colon cancers. Analysis of *Hunk*$^{-/-}$ mice revealed that this kinase is required for metastasis of c-myc–induced mammary tumors but not c-myc–induced primary tumor formation. Similar to c-myc, amplification of the proto-oncogene *HER2/neu* occurs in 10%–30% of breast cancers and is associated with aggressive tumor behavior. By crossing *Hunk*$^{-/-}$ mice with transgenic mouse models for *HER2/neu*-induced mammary tumorigenesis, we report that Hunk is required for primary tumor formation induced by HER2/neu. Knockdown and reconstitution experiments in mouse and human breast cancer cell lines demonstrated that Hunk is required for maintenance of the tumorigenic phenotype in *HER2/neu*-transformed cells. This requirement is kinase dependent and resulted from the ability of Hunk to suppress apoptosis in association with downregulation of the tumor suppressor p27^{kip1}. Additionally, we find that *Hunk* is rapidly upregulated following *HER2/neu* activation in vivo and in vitro. These findings provide what we believe is the first evidence for a role for Hunk in primary tumorigenesis and cell survival and identify this kinase as an essential effector of the HER2/neu oncogenic pathway.

Introduction

In 2011 approximately 1.5 million women will be diagnosed with breast cancer and over 400,000 women will die from this disease, making breast cancer the most common malignancy among women worldwide and the leading cause of cancer mortality (1). Accordingly, understanding the molecular pathways that contribute to the aggressive behavior of human breast cancers is a critical research priority.

We previously isolated the protein kinase Hunk from a mammary tumor in an *MMTV-neu* transgenic mouse (2–4). *Hunk* encodes an approximately 80-kDa protein containing an aminoterminal kinase domain with modest homology to the SNF1/AMPK family of serine-threonine protein kinases, as well as an SNF1 homology region (SNH) that is conserved among AMPK family members (2). SNF1 and its mammalian ortholog, AMPK, regulate cellular metabolism and stress responses (5–8). Members of the AMPK family of protein kinases also play important regulatory roles in a range of processes relevant to tumorigenesis, including proliferation, differentiation, survival, and migration (9–13).

Using targeted deletion in mice, we recently demonstrated that Hunk is dispensable for normal development but is required for the metastasis of mammary tumors induced by the oncogene c-myc (14). Reconstitution experiments demonstrated that Hunk is sufficient to restore the metastatic potential of Hunk-deficient tumor cells, as well as defects in migration and invasion, and does so in a manner that requires its kinase activity (14). Consistent with a role for Hunk in the aggressive behavior of human cancers, elevated expression of this kinase is associated with lymph node–positive and HER2/neu-amplified breast cancers in women and with poorly differentiated carcinomas of the ovary and colon (14). Moreover, a gene expression signature that distinguishes *Hunk* wild-type from *Hunk*-deficient mouse mammary tumors predicts clinical outcome in women with breast cancer (14). Together, these observations suggest that Hunk might play a role in modulating mammary cancer progression and metastatic disease. In contrast, a role for Hunk in primary tumorigenesis has not been established.

To investigate the biological underpinnings of the observed association between HER2/neu amplification and elevated Hunk expression in human breast cancers, we used a genetic approach to probe the role played by Hunk in HER2/neu-induced tumorigenesis. We now report that Hunk is rapidly upregulated following HER2/neu activation in mammary epithelial cells in vivo and in vitro, that Hunk is required for primary tumor formation induced by the HER2/neu pathway, and that the requirement for Hunk in HER2/neu tumorigenesis is kinase dependent and results from the ability of Hunk to suppress apoptosis in association with downregulation of the tumor suppressor p27^{kip1}. Our findings provide what we believe is the first evidence for a role for Hunk in primary tumorigenesis and identify this kinase as an essential effector of the HER2/neu oncogenic pathway in breast cancer.

Results

HER2/neu upregulates Hunk expression. Amplification and overexpression of the receptor tyrosine kinase *HER2/neu* (ErbB2) occurs in 10%–30% of human primary breast cancers and is associated with aggressive tumor behavior and poor prognosis (15–18). Supporting the critical nature of HER2/neu signaling in human breast cancers, therapies that target this molecule, such as trastuzumab (Herceptin), are effective in treating HER2/neu-amplified breast cancers (19–25). Unfortunately, many patients do not respond to trastuzumab therapy, and a large proportion of those who do respond eventually

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: *J Clin Invest.* 2011;121(3):866–879. doi:10.1172/JCI42928.

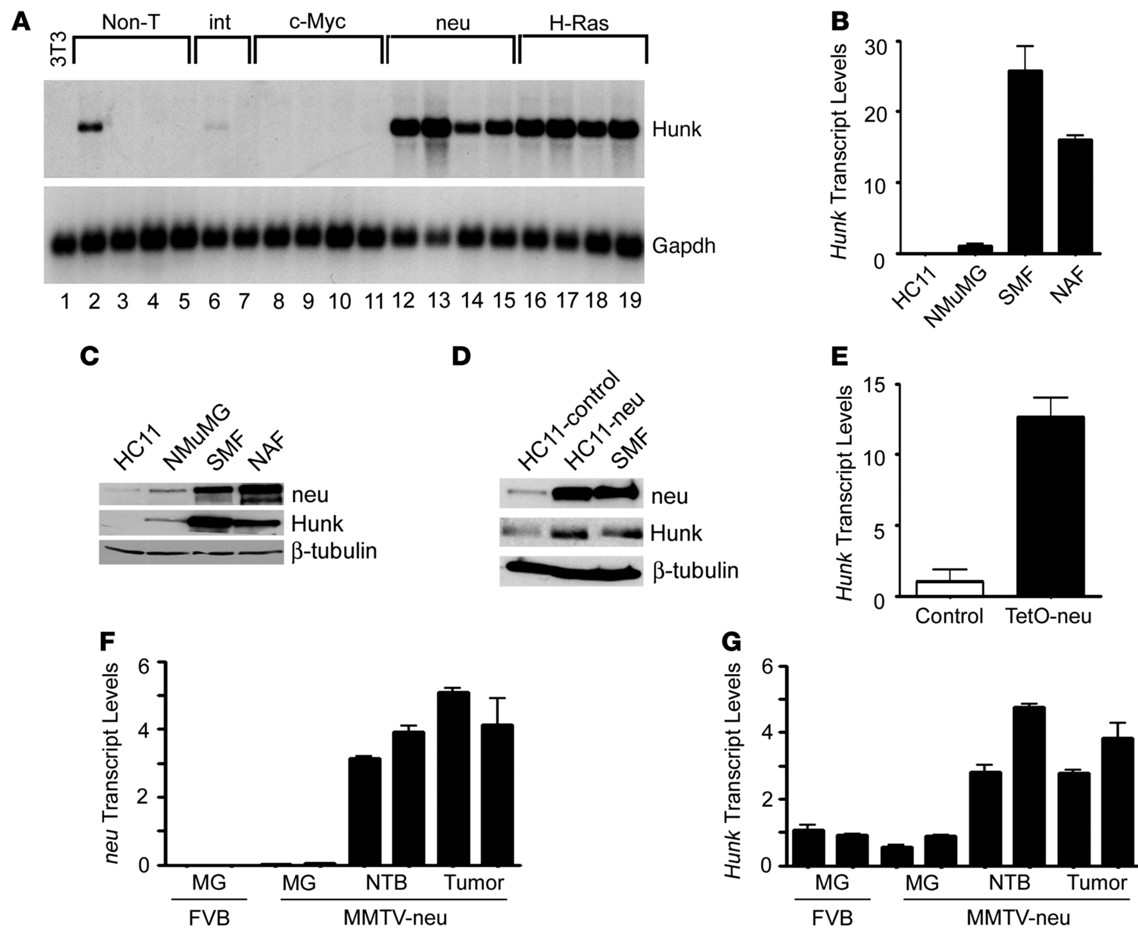

Figure 1

HER2/neu upregulates Hunk. (**A**) Northern blot analysis of Hunk transcript levels in non-transformed mammary cell lines (non-T) and transformed cell lines isolated from tumors derived from *MMTV-Fgf3/int*, *MMTV–c-Myc*, *MMTV-neu*, and *MMTV–H-Ras* mice. (Lanes 1: 3T3; 2: NMuMG; 3: HC11; 4: CL-S1; 5: HBI2; 6: 1128; 7: 8Ma1a; 8: MBp6; 9: M1011; 10: M158; 11: 16MB9a; 12: SMF; 13: NAF; 14: NF639; 15: NF11005; 16: NK-2; 17: AC816; 18: AC711; 19: AC236.) (**B**) *Hunk* levels as determined by QRT-PCR in non-transformed mammary cell lines HC11 and NMuMG and HER2/neu-transformed cell lines SMF and NAF. (**C**) Western blot analysis of Hunk and HER2/neu protein levels in HC11, NMuMG, SMF, and NAF cells. (**D**) Western blot analysis of Hunk protein levels in HC11 cells stably expressing an empty vector control (HC11-control), an activated allele of neu (HC11-neu), or SMF cells. Hunk was immunoprecipitated from 1 mg total protein of each individual cell type. (**E**) *Hunk* transcript levels as determined by QRT-PCR in mammary glands isolated from *MTB/TAN* mice induced with doxycycline for 96 hours, represented as the average level of *Hunk* expression in individual mammary glands ($n = 3$). $P < 0.01$. Data represent mean ± SEM. (**F** and **G**) QRT-PCR analysis of *neu* (**F**) and *Hunk* (**G**) in mammary glands (MG) isolated from wild-type FVB mice or in architecturally normal mammary glands from 10-week-old *MMTV-neu* mice, hyperplastic non–tumor-bearing (NTB) glands from *MMTV-neu* mice, or tumors from *MMTV-neu* mice.

develop resistance (26). Consequently, understanding the signaling molecules regulated by HER2/neu is critical for the development of novel approaches to overcome trastuzumab resistance.

We previously isolated the serine-threonine protein kinase Hunk from a HER2/neu-induced mouse mammary tumor and demonstrated that this kinase is overexpressed in HER2/neu-positive human breast cancers (2, 4, 14). In light of this association, we wished to determine whether Hunk is differentially expressed in mouse mammary tumors induced by different oncogenic pathways. Therefore, we assessed Hunk expression in cell lines established from mouse mammary tumors induced by the HER2/neu, H-ras, c-myc, and Fgf3/int-2 oncogenes and in non-transformed mammary epithelial cells.

Northern hybridization analysis revealed that Hunk is markedly and preferentially overexpressed in cell lines derived from tumors induced by the HER2/neu and H-ras pathways (Figure 1A). In contrast, cell lines derived from tumors induced by the c-myc and Fgf3/int-2 pathways expressed only low levels of Hunk, as did non-transformed cell lines (Figure 1A). Quantitative real-time PCR (QRT-PCR) and immunoblotting confirmed overexpression of Hunk mRNA and protein in SMF and NAF tumor cell lines derived from *MMTV-neu* transgenic mice compared with the non-transformed mammary epithelial cell lines HC11 and NMuMG (Figure 1, B and C). Of note, compared with HC11 cells, NMuMG cells exhibited higher endogenous levels of Hunk as well as HER2/ErbB2 (Figure 1, B and C).

To test the possibility that HER2/neu activation is directly responsible for the elevated levels of Hunk observed in cell lines derived from HER2/neu-induced mammary tumors, we determined the impact of expressing an activated allele of HER2/neu on Hunk expression in HC11 cells (HC11-neu). This revealed that HER2/neu

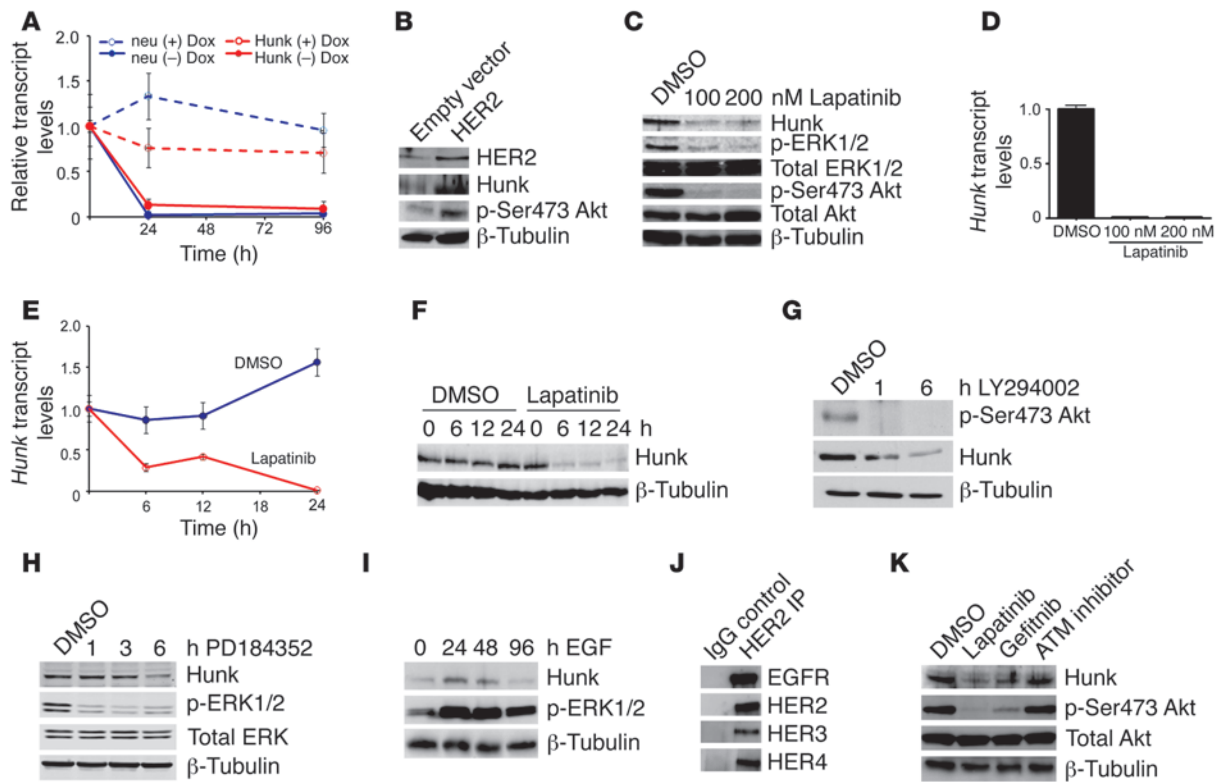

Figure 2

Inhibition of HER2/neu activity downregulates Hunk. (**A**) QRT-PCR analysis of *neu* and *Hunk* in *MTB*/*TAN* cells at 0, 24, and 96 hours after doxycycline removal from growth medium. (**B**) Western blot analysis for Hunk in MCF10A cells transduced with wild-type HER2 or a control vector. (**C**) Western blot analysis of Hunk, phospho-Akt, and phospho-ERK1/2 in BT474 cells treated with DMSO (vehicle) or 100 nM or 200 nM lapatinib for 24 hours. (**D**) QRT-PCR analysis of *Hunk* mRNA levels in BT474 cells treated with DMSO (vehicle) or 100 nM or 200 nM lapatinib for 24 hours. (**E** and **F**) QRT-PCR (**E**) and Western blot (**F**) analysis of Hunk levels in BT474 cells treated with DMSO (vehicle) or 100 nM lapatinib for 0, 6, 12, or 24 hours. (**G**) Western blot analysis of Hunk in SMF cells treated with DMSO (vehicle) or 20 nM LY249002 for 1 or 6 hours. (**H**) Western blot analysis of Hunk in SMF cells treated with DMSO (vehicle) or 1 mM PD184352 for 1, 3, or 6 hours. (**I**) Western blot analysis of Hunk protein levels in BT474 cells stimulated with EGF after 24 hours of serum withdrawal. Cells were stimulated with 5 μg/ml EGF for 24, 48, or 96 hours. (**J**) Western blot analysis of EGFR, HER3, and HER4 bound to immunoprecipitated HER2. (**K**) Western blot analysis of BT474 cells treated with DMSO (vehicle), 100 nM lapatinib, 100 nM gefitinib, or ATM inhibitor (negative control) for 24 hours.

activation resulted in an increase in Hunk protein expression to a level comparable to that found in SMF cells (Figure 1D). Similarly, overexpressing activated HER2/neu in HC11 cells upregulated *Hunk* mRNA expression to a level comparable to that observed in NAF cells (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI42928DS1). Consistent with this, analysis of Hunk and HER2/neu transcript levels confirmed that the ratio of Hunk to HER2/neu expression in HC11-neu and NAF cells was nearly identical (Supplemental Figure 1).

To extend this observation, we used inducible transgenic mice to conditionally express HER2/neu in the mammary gland in response to doxycycline treatment (27–29). This revealed that activation of HER2/neu in the mammary glands of *MMTV-rtTA;TetO-neu* mice for 96 hours resulted in a striking upregulation of *Hunk* mRNA expression (Figure 1E).

To determine the association between HER2/neu and Hunk expression at different stages of mammary tumorigenesis, we used QRT-PCR to quantify *HER2/neu* and *Hunk* mRNA levels in architecturally normal mammary glands, hyperplastic non–tumor-bearing mammary glands, and mammary tumors derived from *MMTV-neu* transgenic mice as well as in mammary glands from wild-type mice (Figure 1, F and G). This revealed that Hunk and HER2/neu are coordinately and comparably upregulated in hyperplastic mammary glands as well as in tumors in *MMTV-neu* mice, which suggests that Hunk and HER2/neu are upregulated in parallel during progressive steps of mammary tumorigenesis.

Together, our results demonstrate that HER2/neu activation rapidly upregulates Hunk at the mRNA and protein levels. This finding, in turn, is consistent with our observation that Hunk is preferentially expressed in HER2/neu-induced mouse mammary tumor cell lines as well as in human breast cancers that overexpress HER2/neu.

Hunk expression requires HER2/neu activity for maintenance. Our observations that Hunk (a) is rapidly upregulated following HER2/neu activation, (b) parallels HER2/neu upregulation during HER2/neu-induced mammary tumorigenesis, and (c) is overexpressed in HER2/neu-induced mouse mammary tumor cell lines and HER2/neu-overexpressing human breast cancers suggested the possibility that HER2/neu activity is required for the maintenance of Hunk expression. To address this hypothesis, we asked whether inhibition of HER2/neu activity would result in the downregulation of Hunk expression. To accomplish this,

Figure 3

Hunk is required for HER2/neu-induced mammary tumorigenesis. (**A**) Tumor-free survival curves comparing *MMTV-neu;Hunk*–wild-type with *MMTV-neu;Hunk*–deficient mice. $P = 0.001$; HR = 2.4. (**B**) Tumor-free survival curves comparing *MTB/TAN;Hunk*–wild-type with *MTB/TAN;Hunk*-deficient mice treated with 0.1 mg/ml doxycycline. $P = 0.0001$; HR 4.4. (**C**) Average number of tumors per animal in *MMTV-neu;Hunk*–wild-type ($n = 22$) and *MMTV-neu;Hunk*–deficient mice ($n = 16$). All 10 mammary glands were examined at necropsy to determine total tumor number per animal. $^*P < 0.01$. Data represent mean ± SEM. (**D**) Average number of tumors per animal in *MTB/TAN;Hunk*–wild-type mice ($n = 12$) and *MTB/TAN;Hunk*-deficient mice ($n = 14$) treated with 0.1 mg/ml doxycycline. $^*P < 0.05$. Data represent mean ± SEM. (**E**) Whole-mount analysis of carmine-stained non–tumor-bearing glands from *MMTV-neu;Hunk*–wild-type and *MMTV-neu;Hunk*–deficient mice. White arrows indicate focal hyperplasias. (**F**) Hematoxylin and eosin staining of a representative hyperplasia (top) and tumor (bottom) from a *MMTV-neu;Hunk*–wild-type mouse. Original magnification, ×6.3 (**E**); ×200 (**F**). (**G**) Quantification of the total number of lesions in non–tumor-bearing glands isolated from *MMTV-neu;Hunk*–wild-type mice and *MMTV-neu;Hunk*–deficient mice. $^*P < 0.05$. Data represent mean ± SEM.

we employed a mammary cell line isolated from a tumor arising in an *MMTV-rtTA;TetO-neu* mouse, in which *HER2/neu* transgene expression is doxycycline dependent (28). As anticipated, removal of doxycycline from the media of *MMTV-rtTA;TetO-neu* tumor cells resulted in the downregulation of *neu* mRNA expression within 24 hours (Figure 2A). Similar to *neu*, *Hunk* mRNA levels were also downregulated within 24 hours of doxycycline withdrawal (Figure 2A). These results suggest that maintenance of *Hunk* expression at the mRNA level requires continued HER2/neu pathway activation.

We next asked whether a similar relationship exists between HER2 activation and Hunk expression in human cells. Human HER2 was expressed in the non-transformed human mammary epithelial cell line MCF10A. Expression of wild-type HER2 in MCF10A cells resulted in upregulation of endogenous human Hunk as well as activation of Akt (Figure 2B). To determine whether HER2 kinase activity is required to maintain human HUNK levels, we next treated the HER2-amplified human breast cancer cell line BT474 with lapatinib, a small molecule inhibitor of HER2. As predicted, lapatinib treatment inhibited HER2 activity, as mea-

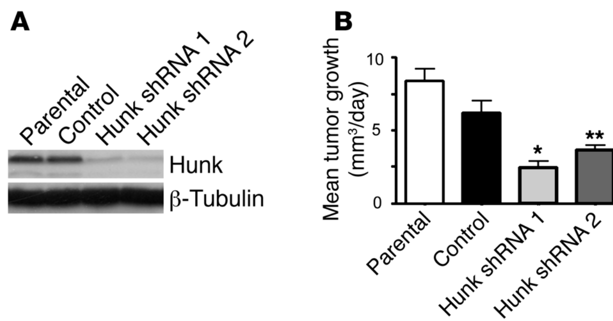

Figure 4
Hunk is required for maintenance of HER2/neu-induced tumor growth. (**A**) Western blot analysis of Hunk protein levels in parental SMF cells and SMF cells expressing a control shRNA, or shRNAs directed against Hunk (shRNA1, shRNA2). (**B**) Mean tumor growth rate of tumors derived from parental SMF cells and SMF cells expressing a control shRNA, Hunk shRNA1, or Hunk shRNA2. $^{*}P < 0.00001$ and $^{**}P < 0.0001$, control versus shRNA1. Data represent mean ± SEM.

sured by decreased activation of Akt and ERK1/2, and was also sufficient to downregulate Hunk at both the mRNA and protein levels within 24 hours (Figure 2, C and D).

To further explore whether regulation of HUNK expression by HER2/neu occurs at the transcriptional level in human cells, we examined *HUNK* mRNA and protein levels in BT474 cells at increasing times following lapatinib treatment. Confirming our prior observations, *HUNK* mRNA levels were dramatically reduced within 6 hours of lapatinib treatment and were nearly absent within 24 hours (Figure 2E). HUNK protein levels declined within a similar time course, with dramatic reductions evident at 6 hours (Figure 2F). Together, these findings demonstrate that Hunk expression requires HER2/neu signaling in human as well as mouse cells and that Hunk is regulated by HER2/neu at the transcriptional level.

HER2/neu activates a number of downstream signaling pathways, including Akt and ERK1/2. Therefore, given our observation that Hunk is transcriptionally regulated by HER2/neu, we next asked whether Hunk expression is dependent upon Akt or ERK1/2 signaling. SMF cells were treated with either the PI3K/Akt pathway inhibitor LY249002 or the MAPK/ERK inhibitor PD184352. As few as 6 hours of treatment with either inhibitor resulted in a decrease in Hunk protein levels (Figure 2, G and H). Therefore, consistent with our finding that Hunk expression requires HER2/neu signaling, Hunk expression levels were also regulated by pathways downstream of HER2/neu in breast cancer cells.

To extend these findings, we next asked whether HUNK expression in human breast cancer cells is regulated by ligands for ErbB family members. BT474 cells were serum deprived for 24 hours and stimulated with EGF for 24, 48, or 96 hours. As anticipated, EGF treatment upregulated levels of phosho-ERK1/2 (Figure 2I). Concomitant with ERK1/2 activation, Hunk protein levels also increased, suggesting that Hunk can be regulated by EGFR signaling or EGFR:HER2 heterodimerization (Figure 2I).

Previous reports have indicated that HER2 heterodimerizes with EGFR and HER3 in BT474 cells and that gefitinib treatment disrupts these interactions (30). Consistent with this, our co-immunoprecipitation experiments confirmed that HER2 stably interacts with EGFR, HER3, and HER4 in BT474 cells (Figure 2J).

To determine whether disruption of HER2 interactions with EGFR family members plays a role in regulating Hunk levels, we treated BT474 cells with 100 nM of the EGFR-selective inhibitor gefitinib. Similar to lapatinib, gefitinib treatment led to the downregulation of HUNK expression (Figure 2K). Together, these findings support a model in which HUNK is transcriptionally regulated by HER2 heterodimerization and activation of EGFR family receptors.

Hunk is required for HER2/neu-induced mammary tumorigenesis. Our observations that HER2/neu activation rapidly upregulates Hunk in vivo and in vitro, and that Hunk is preferentially expressed in HER2/neu-positive human breast cancers and HER2/neu-induced mouse mammary tumor cell lines, suggested that Hunk may play a role in mediating the oncogenic effects of HER2/neu. To test this hypothesis, we interbred *MMTV-neu* transgenic mice and mice bearing a constitutive deletion of *Hunk* (14). Cohorts of *MMTV-neu* mice that were either wild-type or deficient in *Hunk* were generated and monitored for mammary tumor development. In contrast to our observation that Hunk is not required for primary tumorigenesis in *MMTV–c-myc* transgenic mice (14), tumor development was markedly delayed in *Hunk*-deficient *MMTV-neu* mice compared with wild-type *Hunk* mice (hazard ratio [HR] 2.4, $P = 0.001$) (Figure 3A). Consistent with this, tumor multiplicity calculated as the average number of tumors per animal at sacrifice was approximately 50% lower in *Hunk*-deficient *MMTV-neu* mice compared to *Hunk* wild-type *MMTV-neu* mice (Figure 3C; $P = 0.008$).

To confirm these results, we generated cohorts of *MMTV-rtTA;TetO-neu*–inducible transgenic mice that were either *Hunk*–wild-type or *Hunk*–deficient. HER2/neu expression was induced by doxycycline administration at 6 weeks of age, and mice were monitored for mammary tumor development. Similar to findings in *MMTV-neu* mice, mammary tumor development was markedly delayed (T_{50} = 15 weeks vs. 30 weeks, HR 4.4, $P = 0.0001$), and tumor multiplicity was substantially decreased, in *Hunk*-deficient compared with *MMTV-rtTA;TetO-neu;Hunk*–wild-type mice (Figure 3, B and D).

Histopathological analysis of non–tumor-bearing glands from *MMTV-neu* mice revealed the presence of hyperplastic nodules that resembled *MMTV-neu* tumors (Figure 3, E and F). Consistent with our observation that loss of Hunk inhibits HER2/neu-induced mammary tumor formation, the mammary glands of *Hunk*-deficient *MMTV-neu* mice contained fewer hyperplastic nodules than their wild-type counterparts (Figure 3, E and G). Together, our findings demonstrate that Hunk is required for HER2/neu-induced primary mammary tumor formation in vivo.

Hunk is required for maintenance of the tumorigenic phenotype in HER2/neu tumor cells. Our observations that Hunk expression is regulated by HER2/neu activity and is required for HER2/neu-induced mammary tumor formation suggest that Hunk is a downstream effector of HER2/neu. This led us to hypothesize that Hunk might be required for maintenance of the tumorigenic phenotype in HER2/neu-transformed cells. If true, this hypothesis would predict that Hunk downregulation would mimic the effects of HER2/neu-targeted therapy. In this regard, while our results in *MMTV-neu* and *MMTV-rtTA;TetO-neu* mice demonstrated that Hunk is required for HER2/neu-induced tumor formation, they could not rule out the possibility that Hunk is required for the development of HER2/neu-induced mammary tumors, but not their maintenance.

To determine whether Hunk is required for maintenance of the tumorigenic phenotype in HER2/neu tumor cells, we generated SMF cells stably expressing 2 different shRNAs targeted against

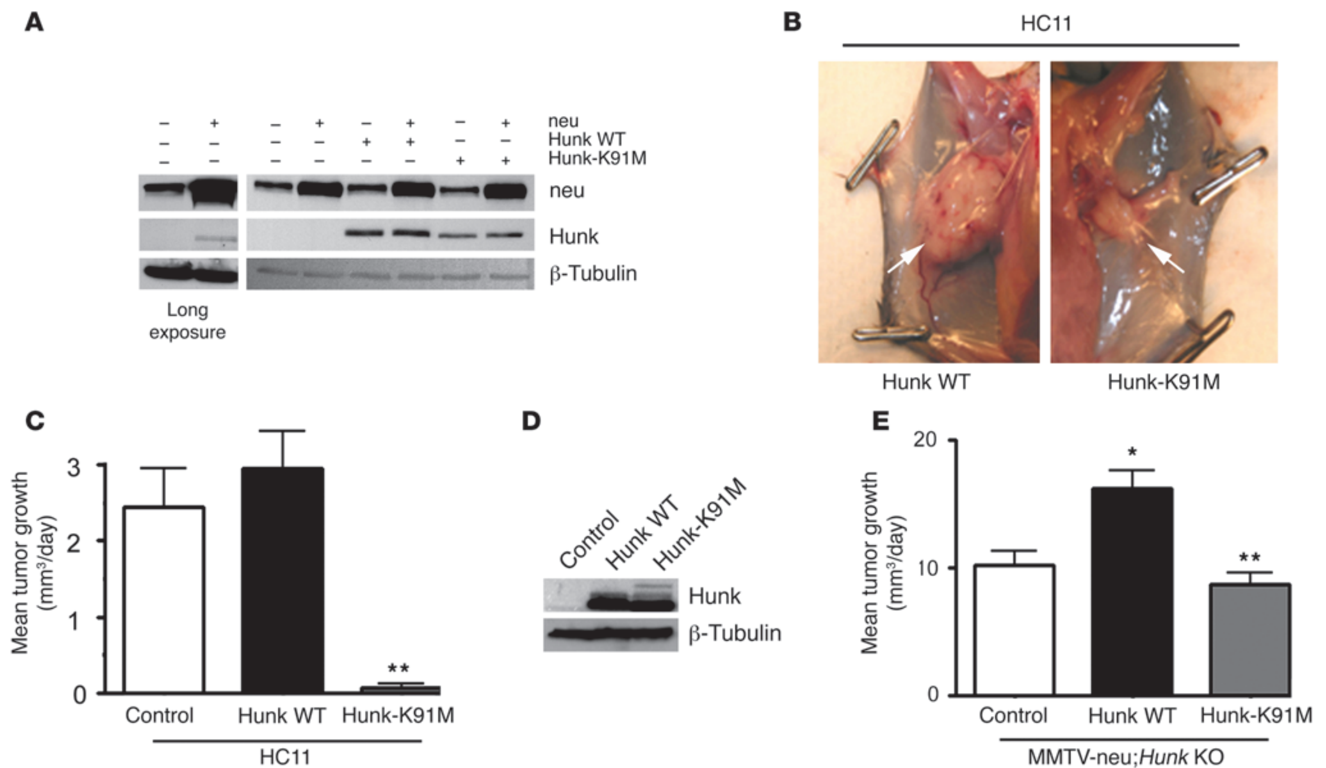

Figure 5

Expression of a kinase-dead allele of Hunk impairs growth of HER2/neu-induced tumors. (**A**) Western blot analysis confirming expression of HER2/neu, Hunk–wild-type, and Hunk-K91M in HC11 cells. Left panel shows a longer autoradiographic exposure to illustrate the previously observed increase in endogenous Hunk levels in empty vector cells expressing HER2/neu. (**B**) Gross comparison of tumors derived from HC11;Hunk–wild-type and HC11;Hunk-K91M cells sacrificed at the same time after injection. Average volume of wild-type Hunk–derived tumors was 237.9 mm^3, compared with 15.5 mm^3 for Hunk-K91M–derived tumors. White arrows indicate tumors. (**C**) Mean tumor growth rates of tumors derived from HC11;vector control–, HC11;Hunk–wild-type–, or HC11;Hunk-K91M–expressing cells. **$P < 0.01$, Hunk–wild-type versus Hunk-K91M. Data represent mean ± SEM. (**D**) Western blot analysis for Hunk in *MMTV-neu;Hunk*–deficient cells reconstituted with vector control, Hunk–wild-type, or Hunk-K91M. (**E**) Mean tumor growth rate of orthotopic tumors derived from *MMTV-neu;Hunk*–deficient cells reconstituted with Hunk–wild-type, Hunk-K91M, or vector control. *$P < 0.01$, control versus Hunk–wild-type; **$P < 0.001$, Hunk–wild-type versus Hunk-K91M. Data represent mean ± SEM.

Hunk or a control shRNA targeted against firefly luciferase. Stable transduction of *Hunk* shRNA1 or shRNA2 reduced Hunk protein levels compared with the parental SMF cells, whereas the control shRNA did not (Figure 4A).

Orthotopic transplantation of each of these SMF-derived cell lines into the mammary fat pads of *nu/nu* mice revealed that SMF cells in which Hunk expression had been knocked down exhibited mean tumor growth rates that were approximately half that of those observed for parental or control shRNA-expressing SMF cells (Figure 4B; $P = 0.0006$ and $P = 0.009$, respectively). Mean tumor growth rates of control shRNA-expressing cells did not differ from parental cells. Since downregulating Hunk in fully transformed HER2/neu tumor cells impairs their growth as tumors, these findings suggest that Hunk is required for maintenance of the tumorigenic phenotype of HER2/neu-expressing breast cancer cells.

Hunk kinase activity is required for growth of HER2/neu-expressing tumors. We previously demonstrated that Hunk kinase activity is required for the migration, invasion, and metastasis of myc-induced mammary tumor cells (14). Therefore, in an analogous manner, we asked whether Hunk kinase activity is required for the growth of HER2/neu-induced mammary tumors. To address this, we stably transduced non-transformed HC11 cells that express low levels of Hunk with vectors expressing either Hunk–wild-type or a point mutant allele of Hunk, Hunk-K91M, which abolishes Hunk kinase activity (31–33). Immunoblotting demonstrated that HC11–Hunk–wild-type and HC11–Hunk-K91M cells expressed comparable levels of Hunk (Figure 5A).

Next, we transformed Hunk–wild-type– and Hunk-K91M–expressing cells with an oncogenic allele of HER2/neu. Following orthotopic transplantation into the mammary glands of *nu/nu* mice, HC11 cells expressing either wild-type or K91M Hunk did not form tumors (data not shown), indicating that overexpression of Hunk alone is not tumorigenic. Notably, tumors derived from neu-transformed HC11 cells that had been transduced with Hunk-K91M were dramatically smaller than their wild-type Hunk counterparts when assessed at the same post-injection time point (Figure 5B). Quantification of mean tumor growth rates revealed that neu-transformed Hunk-K91M–expressing cells grew at a rate that was only 4% of that of HER2/neu-transformed HC11 cells transduced with Hunk wild-type (Figure 5C; $P < 0.0001$). HC11 cells transduced with HER2/neu and wild-type Hunk grew at the same rate as HC11 cells transduced with HER2/neu alone, consistent with our finding that HC11 cells transduced with HER2/neu express endogenous Hunk.

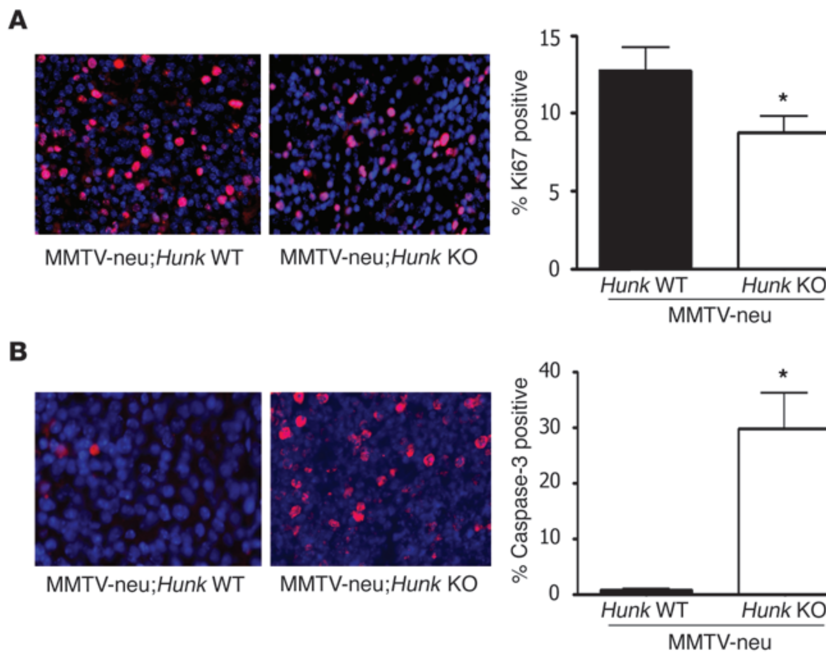

Figure 6
Hunk is required for cell proliferation and survival in HER2/neu-induced mammary tumors. (**A**) *MMTV-neu;Hunk*–wild-type and *MMTV-neu;Hunk*–deficient tumors were stained with Ki67 as a marker of proliferation. Levels of Ki67 staining were quantified by measuring the percentage of positive-staining nuclei. $n = 4$ individual tumors per genotype. $^{*}P < 0.05$. Data represent mean ± SEM. (**B**) *MMTV-neu;Hunk*–wild-type and *MMTV-neu;Hunk*–deficient tumors were stained with cleaved caspase-3 as a marker of apoptosis. Levels of cleaved caspase-3 staining were quantified by measuring the percentage of positive-staining nuclei. $n = 4$ individual tumors per genotype. $^{*}P < 0.001$. Data represent mean ± SEM. Original magnification, ×200.

To extend these findings by eliminating any potential effects of endogenous Hunk, we next isolated cells from *MMTV-neu*;*Hunk*–deficient tumors and reconstituted these cells with wild-type Hunk, a K91M allele of *Hunk*, or an empty vector control (Figure 5D). *MMTV-neu*;*Hunk*–deficient tumor cells reconstituted with wild-type Hunk displayed an approximately 2-fold increase in mean tumor growth rate compared with control cells lacking Hunk (Figure 5E). In contrast, *MMTV-neu*;*Hunk*–deficient tumor cells reconstituted with Hunk-K91M exhibited tumor growth rates indistinguishable from control cells lacking Hunk (Figure 5E). These findings indicate that Hunk kinase activity is required for maintenance of the tumorigenic phenotype of HER2/neu-expressing tumor cells and further suggest that Hunk-K91M acts as a dominant-negative allele in the presence of wild-type Hunk.

Hunk is required for survival of HER2/neu-expressing tumor cells. HER2/neu downregulation or inhibition with pharmacological agents such as trastuzumab induces tumor regression via mechanisms associated with cell cycle arrest and apoptosis (29, 34). In light of our findings indicating that Hunk mediates at least some of the oncogenic effects of HER2/neu, we reasoned that loss of Hunk in HER2/neu-expressing cells might have effects similar to those observed following pharmacological inhibition of HER2/neu.

To test this hypothesis, we quantified cellular proliferation and apoptosis rates in mammary tumors arising in *Hunk* wild-type and *Hunk*-deficient *MMTV-neu* mice. Whereas immunofluorescence analysis detected a modest, approximately 33% decrease in the percentage of Ki67-positive cells in *Hunk*-deficient *MMTV-neu* tumors (Figure 6A; $P = 0.03$), immunofluorescence for cleaved caspase-3 revealed a dramatic, approximately 30-fold increase in levels of apoptosis in HER2/neu-induced tumors arising in *Hunk*-deficient mice (Figure 6B; $P = 0.0002$). These findings suggest that promotion of cell survival may constitute an essential function of Hunk in HER2/neu-expressing mammary tumors.

Hunk regulates $p27^{kip1}$ expression and localization. $p27^{kip1}$ is a Cdk inhibitor whose expression leads to G1 cell cycle arrest by inhibiting cyclin E/Cdk2 activity (35). p27 functions as a haplo-insufficient tumor suppressor in mice, and its levels are reduced in human breast cancer (36, 37). Reduced p27 levels are also associated with HER2/neu overexpression and poor prognosis in human cancers, including breast cancer (38–40). A mechanistic basis for this association is suggested by the finding that HER2/neu overexpression in human breast cancer cells reduces p27 stability and promotes cell survival (41). Conversely, treatment of breast cancer cell lines with HER2/neu or EGFR inhibitors enhances p27 stability (42, 43), which in turn results in cell cycle arrest and apoptosis (44–49). Consistent with the importance of p27 downregulation in HER2/neu-induced tumorigenesis, heterozygous deletion of p27 in *MMTV-neu* mice accelerates mammary tumorigenesis and mammary glands from these mice exhibit increased proliferation and decreased apoptosis (50). Conversely, p27 overexpression in HER2/neu-expressing fibroblasts inhibits focus formation in vitro and tumor formation in vivo (51). Moreover, downregulation of p27 confers resistance to anti-estrogens in vitro, and women whose breast cancers express low levels of p27 respond poorly to anti-estrogen therapy (52, 53).

In light of our observations that tumorigenesis is delayed in Hunk-deficient mice and that Hunk-deficient HER2/neu-induced tumors exhibit increased levels of apoptosis, we hypothesized that the requirement for Hunk in HER2/neu-induced mammary tumorigenesis might be due to downregulation of p27. To address this hypothesis, we assessed p27 protein levels in SMF cells expressing shRNAs directed against Hunk. This revealed that Hunk knockdown in HER2/neu tumor cells resulted in increased levels of p27 (Figure 7A). Consistent with reports that total p27 levels and levels of serine-10 phosphorylated p27 are increased in trastuzumab-treated cells, Hunk knockdown in SMF cells resulted in the upregulation of both p27 and serine 10–phosphorylated p27 (Figure 7A).

Modulation of p27 expression is principally posttranslational and occurs via regulation of p27 nuclear export (43). Consistent with this, treatment of HER2/neu-expressing breast cancer cells with trastuzumab promotes p27 nuclear accumulation in asso-

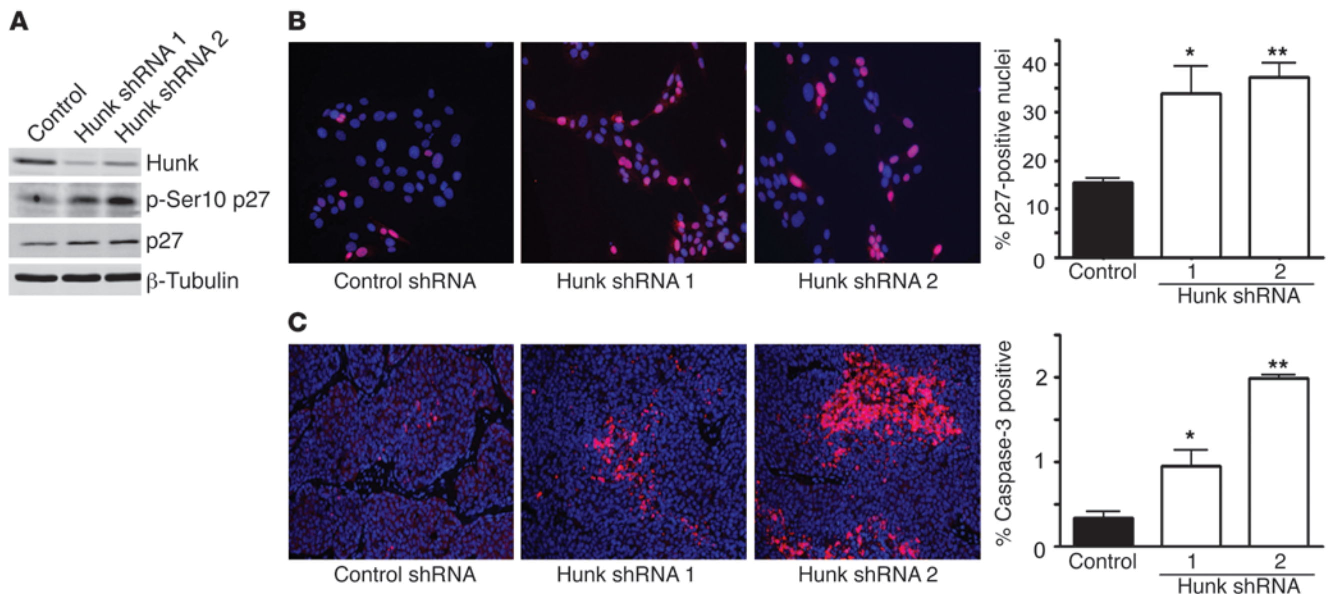

Figure 7

Hunk negatively regulates p27 expression and localization in vitro. (**A**) Western blot analysis of SMF cells expressing shRNAs directed against Hunk reveal elevated total p27 protein levels and increased phosphorylation on serine 10 of p27 compared with cells expressing control shRNA. (**B**) SMF cells expressing either control shRNA or shRNAs directed against Hunk were stained for p27 by immunofluorescence. Quantification of nuclear p27 staining was determined by measuring the percentage of positive-staining nuclei. $^{*}P < 0.01$, $^{**}P < 0.0001$ versus control. Data represent mean ± SEM. (**C**) Immunofluorescence for cleaved caspase-3 in tumors derived from SMF cells expressing either a control shRNA or shRNAs directed against Hunk. $n = 3$ individual tumors per genotype. $^{*}P < 0.05$, $^{**}P < 0.0001$ versus control. Data represent mean ± SEM. Original magnification, ×200 (**B**); ×50 (**C**).

ciation with increased p27 protein levels, increased serine 10 phosphorylation, and increased apoptosis (41, 44–47, 54–56). To determine whether Hunk affects p27 subcellular localization, we performed immunofluorescence for p27 in SMF cells expressing a control shRNA or shRNAs directed against Hunk. Hunk knockdown resulted in a 2.3-fold increase in the percentage of cells exhibiting p27 nuclear localization (Figure 7B). This finding is consistent with a model in which Hunk is required for nuclear export of p27 in HER2/neu-transformed cells.

Avoiding apoptosis is a hallmark of cancer, and downregulation of p27 is one mechanism by which tumor cells can achieve this goal (48, 49, 56–58). Consistent with this, low levels of p27 are associated with poor prognosis in a number of human cancers, including breast cancer (38, 40, 48, 55). Given our observations that p27 levels and nuclear localization are upregulated in HER2/neu-transformed tumor cells in which Hunk expression has been knocked down, and that Hunk-deficient tumors in *MMTV-neu* mice exhibit increased levels of apoptosis, we reasoned that downregulating Hunk in neu-induced tumors would induce apoptosis.

To test this hypothesis, we performed immunofluorescence for cleaved caspase-3 in tumors generated by orthotopic transplantation of SMF cells expressing either control shRNAs or shRNAs directed against Hunk. Consistent with our findings in intact mice, tumors derived from SMF cells expressing Hunk shRNAs exhibited an approximately 2- to 4-fold increase in apoptosis compared with control tumors (Figure 7C; $P = 0.04$ and $P < 0.0001$, respectively). Tumors derived from SMF cells expressing Hunk shRNAs also exhibited increased levels of nuclear p27, consistent with our in vitro observations (Supplemental Figure 2). In aggregate, our findings indicate that Hunk is required for the maintenance of HER2/neu–expressing mammary tumor cells by virtue of its ability to act as a pro-survival factor.

Hunk regulates p27^{kip1} levels and localization downstream of HER2/neu activation. To further extend our in vitro and in vivo findings in SMF cells to a model for spontaneous HER2/neu-induced mammary tumorigenesis, we evaluated total p27 levels in *MMTV-neu* tumors isolated from *Hunk*–wild-type and *Hunk*-deficient mice. As predicted, *Hunk*-deficient tumors exhibited significantly elevated levels of p27 compared with *Hunk*–wild-type tumors (Figure 8A). Eight of 11 Hunk-deficient tumors exhibited high p27 protein levels, defined as levels at least 2-fold greater than the lowest detectable amount of p27, whereas only 3 of 9 *Hunk*–wild-type tumors exhibited high levels of p27 ($P < 0.05$, Chi-squared test; Figure 8A and data not shown). Overall, mean p27 levels were 2-fold higher in HER2/neu-induced *Hunk*-deficient tumors compared with *Hunk*–wild-type tumors (Figure 8B). Moreover, consistent with our in vitro findings in SMF cells, tumors with high p27 levels also displayed elevated levels of serine 10 phosphorylation (Figure 8A).

To determine whether Hunk kinase activity is required for downregulating p27 expression and localization in the context of HER2/neu activation, we isolated tumor cells from *Hunk* wild-type and *Hunk*-deficient *MMTV-neu* tumors. Next, we generated an isogenic set of cell lines by reconstituting *MMTV-neu*;*Hunk*–deficient cells with wild-type Hunk, a kinase-inactive Hunk-K91M allele, or an empty vector control. Consistent with our in vitro findings, p27 levels were elevated in *MMTV-neu*;*Hunk*–deficient tumors cells compared with *MMTV-neu*;*Hunk*–wild-type tumor cells (Figure 8C). Moreover, re-expression of Hunk wild-type in *Hunk*-deficient

Figure 8
Hunk negatively regulates p27 expression and localization in vivo. (**A**) Western blot analysis of p27 and phospho–serine 10 in *MMTV-neu;Hunk*–wild-type and *MMTV-neu;Hunk*–deficient tumor samples. (**B**) Quantification of p27 protein levels from 9 *MMTV-neu;Hunk*–wild-type tumors and 11 *MMTV-neu;Hunk*–deficient tumors. $^{*}P < 0.05$. Data represent mean ± SEM. (**C**) Western blot analysis for p27 in *MMTV-neu;Hunk*–wild-type tumor cells and *MMTV-neu;Hunk*–deficient tumors cells reconstituted with vector control, Hunk wild-type, or Hunk-K91M. (**D**) Immunofluorescence for p27 in *MMTV-neu;Hunk*–wild-type tumor cells and *MMTV-neu;Hunk*–deficient tumors cells reconstituted with vector control, Hunk–wild-type, or Hunk-K91M. Data represent mean ± SEM. $^{*}P < 0.05$, versus *MMTV-neu;Hunk*–wild-type. $^{**}P < 0.05$, versus *MMTV-neu;Hunk*–deficient Hunk wild-type. Data represent mean ± SEM. (**E**) Mammary glands from *MTB/TAN;Hunk* wild-type–and *MTB/TAN;Hunk*-deficient mice induced with doxycycline for 96 hours stained by immunofluorescence to assess p27 localization. White arrows indicate CK8-positive cells containing nuclear p27. Original magnification, ×200.

MMTV-neu tumor cells resulted in downregulation of p27, whereas reconstitution with Hunk-K91M did not (Figure 8C). These findings demonstrate that Hunk downregulates p27 in a manner that requires Hunk kinase activity.

To assess the effect of Hunk kinase activity on p27 localization, we performed immunofluorescence for p27 in the 4 cell lines described above. Analogous to our findings in SMF cells expressing Hunk shRNAs, we found that the percentage of tumor cells positive for nuclear p27 was greater in *MMTV-neu*;*Hunk*–deficient vector control cells compared with *MMTV-neu*;*Hunk*–wild-type cells (Figure 8D). Furthermore, re-expression of Hunk wild-type in *MMTV-neu*;*Hunk*–deficient tumor cells resulted in a decrease in the percentage of cells with nuclear p27, whereas reconstitution with Hunk-K91M did not (Figure 8D). Together, these findings demonstrate that Hunk kinase activity is required for the suppression of nuclear p27 accumulation in HER2/neu-transformed tumor cells.

To further examine the ability of Hunk to regulate p27 levels and localization in the context of HER2/neu activation in vivo, we employed bi-transgenic *MMTV-rtTA;TetO-neu* mice. *MMTV-rtTA;TetO-neu*;*Hunk*–wild-type and *MMTV-rtTA;TetO-neu*;*Hunk*–deficient mice were treated with doxycycline for 96 hours to induce expression of activated HER2/neu throughout the mammary epithelium. Mammary glands were isolated from doxycycline-treated animals, and immunofluorescence for p27 was performed. This revealed increased expression and nuclear localization of p27 in *Hunk*-deficient mammary epithelial cells

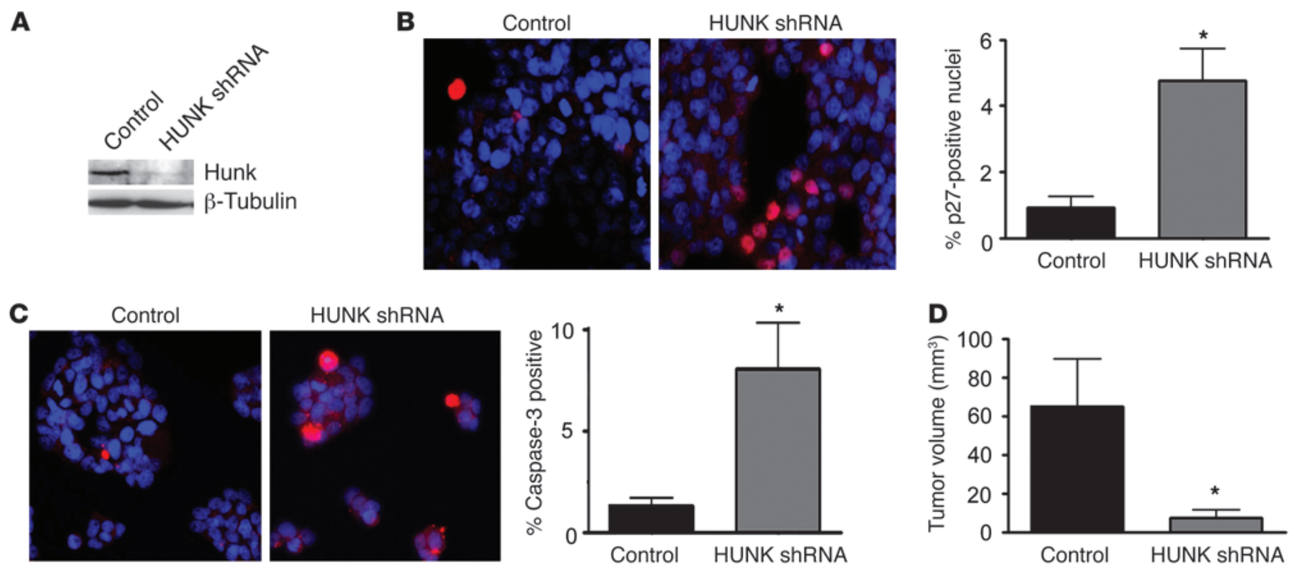

Figure 9

Hunk inhibits tumor formation of human breast cancer cells by modulating p27 localization and cell survival. (**A**) Western blot analysis of Hunk protein levels in BT474 cells expressing a control shRNA or shRNA directed against *Hunk*. (**B**) Immunofluorescence for p27 in BT474 cells expressing control shRNA or Hunk shRNA. $^*P < 0.01$. Data represent mean ± SEM. (**C**) Immunofluorescence for cleaved caspase-3 in BT474 cells expressing control shRNA or Hunk shRNA. $^*P < 0.05$. Data represent mean ± SEM. Original magnification, ×200. (**D**) Average tumor volume was calculated 7 weeks after injection of BT474 cells expressing control shRNA or Hunk shRNA. $^*P < 0.05$. Data represent mean ± SEM.

following acute HER2/neu activation in vivo (Figure 8E). These findings indicate that Hunk negatively regulates p27 expression and nuclear localization in non-transformed mammary epithelial cells in vivo and strongly suggest that Hunk mediates the negative regulatory effects of HER2/neu on p27.

Hunk inhibits the tumorigenic phenotype of human breast cancer cells by modulating p27 and cell survival. Our findings that Hunk is required for the negative regulation of p27 expression and nuclear localization, as well as for the growth and survival of *MMTV-neu* tumor cells (Figure 5E), strongly support the hypothesis that the requirement for Hunk in HER2/neu-induced tumorigenesis is due, at least in part, to the ability of Hunk to promote cell survival by downregulating the levels and nuclear localization of p27. To determine whether our findings were relevant to human breast cancers, we knocked down Hunk in the HER2/neu-amplified human breast cancer cell line, BT474, by expressing shRNAs targeted against human Hunk (Figure 9A). Specifically, we asked whether downregulation of Hunk in BT474 cells would result in an increase in p27 nuclear localization similar to that observed in our murine in vitro and in vivo model systems.

Notably, inhibition of HER2 in BT474 cells by treatment with lapatinib or gefitinib results in increased nuclear localization of p27 and increased apoptosis (30). In an analogous manner, our immunofluorescence staining revealed that Hunk downregulation in BT474 cells resulted in an increase in the percentage of cells with nuclear p27 (Figure 9B). Moreover, consistent with our findings in tumors derived from *MMTV-neu*;*Hunk*–deficient mice or SMF cells in which Hunk had been knocked down by shRNA, BT474 cells expressing shRNAs targeted against human Hunk displayed increased apoptosis, as evidenced by increased staining for cleaved caspase-3 (Figure 9C).

Our findings that Hunk downregulation resulted in increased nuclear p27 and increased apoptosis in BT474 cells expressing Hunk shRNAs led us to predict that these cells would exhibit impaired tumor growth. Therefore, we injected BT474 cells expressing control or Hunk shRNAs into the mammary fat pads of *nu/nu* mice and monitored them for tumor formation. As predicted, Hunk knockdown cells exhibited impaired tumor growth, as BT474 cells expressing a control shRNA grew at a rate that was 6-fold higher than BT474 cells expressing an shRNA targeted against Hunk (Figure 9D). Taken together, our results demonstrate that Hunk plays a similar role in regulating p27 levels, p27 nuclear accumulation, apoptosis, and tumor growth in human and mouse HER2/neu-transformed breast cancer cells.

Discussion

Consistent with its role as a dominant oncogenic pathway in human breast cancer, amplification and overexpression of HER2/neu is associated with aggressive tumor behavior and poor prognosis (15–18), and therapeutic agents that target HER2/neu, such as trastuzumab, are effective in treating this subset of breast cancer patients (19–25). Unfortunately, many breast cancer patients with HER2/neu overexpression do not respond to trastuzumab therapy, and the vast majority of those who do respond to this agent ultimately develop resistance (26). Mechanisms for resistance include molecular alterations in HER2/neu, alterations in the regulation of downstream signaling components, and crosstalk with other pathways that can compensate for attenuated HER2/neu signaling. Accordingly, approaches to overcoming trastuzumab resistance have focused on targeting alternate HER2/neu epitopes and on the combined inhibition of multiple signaling components that either influence HER2/neu signaling or are downstream of HER2/neu (26). The success of these approaches, however, will require elucidating the full complement of signaling pathways downstream of HER2/neu as well as the mechanisms by which this potent oncogene drives the aggressive behavior of human breast cancers.

Our observations that Hunk is preferentially expressed in HER2/neu-induced mouse mammary tumor cell lines, that Hunk is overexpressed in HER2/neu-positive human breast cancers, that HER2/neu activation rapidly upregulates Hunk in vivo and in vitro, and that HER2/neu downregulation or inhibition results in rapid Hunk downregulation, led us to hypothesize that Hunk is a downstream effector of HER2/neu-induced mammary tumorigenesis. To test this hypothesis, we bred *Hunk*-deficient mice to transgenic mice that activate HER2/neu in the mammary epithelium. In both constitutive and inducible transgenic mouse models for mammary-specific HER2/neu activation, mice bearing targeted deletions in *Hunk* displayed decreased tumor incidence, increased tumor latency, and decreased tumor multiplicity. Furthermore, knockdown of Hunk expression in both mouse and human HER2/neu-transformed cell lines, as well as overexpression of a kinase-dead allele of Hunk in cells expressing wild-type Hunk, impaired orthotopic tumor formation following transplantation in mice. Conversely, the growth of tumors derived from *Hunk*-deficient *MMTV-neu* cell lines was rescued by reconstitution with wild-type, but not kinase-dead, Hunk. In aggregate, these findings demonstrate that Hunk expression and kinase activity are required for maintenance of the tumorigenic phenotype in HER2/neu-transformed mammary tumor cells, and that Hunk-deficient tumor cells exhibit a cell-intrinsic defect in tumor growth. Our results identify Hunk as an essential effector of the HER2/neu oncogenic pathway in breast cancer and suggest that pharmacologic inhibition of Hunk may represent a clinically useful approach to the treatment of this aggressive subtype of human breast cancers.

Notably, mammary tumors arising in *Hunk*-deficient *MMTV-neu* mice exhibit markedly elevated rates of apoptosis, as do orthotopic tumors formed from SMF cells or HER2/neu-amplified BT474 human breast cancer cells in which Hunk expression has been knocked down. Taken together, findings in 5 model systems — *MMTV-neu* mice, *MMTV-rtTA;TetO-neu* mice, SMF cells, HER2/neu-transformed HC11 cells, and HER2/neu-amplified human BT474 breast cancer cells — support a model in which Hunk is required to mediate the pro-survival effects of HER2/neu in mammary epithelial cells in vivo.

It has previously been shown that EGFR-expressing HER2-amplified breast cancer cell lines, such as BT474, are sensitive to EGFR inhibition by gefitinib (30). This suggests that combined EGFR/HER2-targeted inhibition may be highly effective. Our observations that EGF treatment upregulates Hunk in BT474 cells and that EGFR inhibition downregulates Hunk suggests that HER2 heterodimerization with EGFR or EGFR homodimerization may contribute to Hunk regulation. As such, our results suggest that Hunk inhibition may be useful in treating HER2-amplified breast cancers, especially those expressing EGFR.

HER2/neu signaling activates multiple downstream pathways, several of which have been shown to be required for HER2/neu-induced mammary tumorigenesis (54, 59–76). Of particular relevance, crossing *MMTV-neu* mice into a $p27^{kip1}$-deficient background revealed a dual role for p27 in HER2/neu-induced mammary tumorigenesis in vivo. Consistent with its established role as a tumor suppressor, loss of one allele of *p27* accelerated tumorigenesis (40). In contrast, loss of both alleles delayed tumor formation due to the essential role of p27 in cell cycle progression, a finding that is consistent with observations that p27 is frequently downregulated, but rarely lost outright, in human cancers (40, 55, 77). Consistent with a requirement for p27 downregulation in HER2/neu tumorigenesis, inhibition of HER2 with targeted therapies results in an increase in the stability and nuclear localization of p27, ultimately resulting in cell death (30, 42, 43). Conversely, reduction of p27 levels in breast cancer cells in vitro confers resistance to chemotherapeutic agents, and low p27 expression is associated with poor response to anti-estrogen therapy in breast cancer patients (52, 53).

We attribute the increase in apoptosis induced by downregulation or loss of Hunk in HER2/neu-transformed tumor cells to the fact that Hunk downregulation results in increased levels and nuclear accumulation of p27, as well as increased levels of serine 10–phosphorylated p27. Serine 10 phosphorylation of p27 in early G1 results in dissociation of p27 from cyclin E1/Cdk2 complexes and facilitates CRM1-dependent nuclear export and degradation of p27 (78–82). Conversely, stabilization of p27, phosphorylation of p27 on serine 10, and nuclear accumulation of p27 in breast cancer cells is strongly associated with apoptosis, particularly in the context of HER2/neu inhibition (44, 49, 56, 57). In aggregate, our observations suggest that the requirement for Hunk in HER2/neu-induced tumorigenesis, as well as the survival of HER2/neu-transformed cells, is due at least in part to the role of Hunk in mediating the negative regulatory effects of HER2/neu on p27.

Our previous finding that elevated Hunk expression is associated with lymph node–positive breast cancers in women suggested the possibility that Hunk may play a role in breast cancer progression (14). Consistent with this, we found that targeted deletion of *Hunk* in mice markedly impairs the metastasis, migration, and invasion of c-myc–induced mammary tumors (8). Moreover, similar to our observations in *MMTV-neu* mammary tumor cells, Hunk kinase activity was required for these effects. However, in contrast to our observations regarding the role of Hunk in HER2/neu-induced primary tumorigenesis, *Hunk* deletion did not impair primary tumorigenesis in *MMTV-c-myc* mice. Consistent with this oncogene-specific effect, Hunk expression was upregulated in HER2/neu–transformed, but not c-myc–transformed, mammary tumor cell lines. Taken together, our findings suggest that Hunk may have a context-dependent and oncogene-specific role in breast cancer. In this regard, substantial differences exist in the histopathology of c-myc– and HER2/neu-induced mouse models of breast cancer. Specifically, while the majority of HER2/neu-induced tumors are categorized as luminal, gene expression profiling has revealed that myc-induced tumors fall into both basal and luminal subtypes (83–86). As such, additional studies will be required to fully elucidate the mechanisms underlying the oncogene- and cell type–specific effects of Hunk on cancer progression.

In this regard, in contrast to our genetic and biochemical evidence for pro-tumorigenic effects of Hunk in HER2/neu-induced tumorigenesis and pro-metastatic effect of Hunk in c-myc–induced mammary tumorigenesis, it has recently been suggested by Mak and colleagues that Hunk might play an opposing role in basal-like human breast cancers, wherein Hunk overexpression may suppress metastatic potential (87). While the findings of that study are formally consistent with the possibility that the role of Hunk in metastasis may be cell type specific, it is important to note that their study focused primarily on in vitro systems in which Hunk was exogenously overexpressed in cell lines that do not otherwise express Hunk. As such, the interpretation and conclusions from these studies must be considered

carefully. In particular, whether this represents a bona fide finding or is instead an artifact of overexpression will await future genetic studies with appropriate animal models.

Finally, the successful clinical application of targeted protein kinase inhibitors, such as imatinib, erlotinib, and trastuzumab, for the treatment of human malignancies reflects the key roles that protein kinases frequently play in the pathogenesis of human cancer. These and other observations have generated intense interest in developing a broad spectrum of targeted therapeutics capable of specifically inhibiting individual kinases (21, 88–92). Ultimately, elucidating the broad range of molecular alterations that occur during breast cancer progression will facilitate targeting of the multiple synergistic pathways that contribute to neoplastic progression and enhance the development of therapeutic agents tailored against the more aggressive forms of this disease.

Methods

Cell culture. Cell lines were grown at 37°C in 5% CO_2 as previously described (2, 93). HC11 cells were maintained in RPMI media (Gibco) supplemented with 10% Super Calf Serum (SCS; Gemini Bio Products), 5 μg/ml insulin (Sigma-Aldrich), 10 ng/ml EGF (Sigma-Aldrich), 200 nM glutamine (Gibco), and penicillin/streptomycin. SMF cells were maintained in DMEM (MediaTech), 10% FBS (Sigma-Aldrich), 5 μg/ml insulin, 200 nM glutamine, and penicillin/streptomycin. NMuMG cells were maintained in DMEM, 10% SCS, 200 nM glutamine, and penicillin/streptomycin. NAF cells were maintained in DMEM (MediaTech), 10% FBS (Gibco), 200 nM glutamine (Gibco), and penicillin/streptomycin. BT474 cells were maintained in RPMI media (Gibco) supplemented with 10% FBS, 200 nM glutamine, and penicillin/streptomycin. Lapatinib was purchased from Life-Sciences (via B-Bridge International). Gefitinib was purchased from LC International.

Animal and tissue preparation. Animal care and all animal experiments were performed with the approval of, and in accordance with guidelines of the University of Pennsylvania IACUC. Mice were housed under barrier conditions with 12-hour light/12-hour dark cycles. For histological analysis, tumors were fixed in 4% paraformaldehyde overnight, followed by incubation in 70% ethanol before paraffin embedding. The resulting paraffin sections were used for staining with hematoxylin and eosin or analysis by immunofluorescence.

Tumorigenesis assay. Hunk-deficient mice were crossed to MMTV-neu mice or MMTV-rtTA (MTB) and TetO-neu (TAN) mice. MMTV-neu female mice of each Hunk genotype were monitored twice weekly for mammary tumors. MTB/TAN female mice of each Hunk genotype were administered doxycycline in their drinking water beginning at 6 weeks of age and were monitored for tumor formation twice weekly. Mice possessing tumors with a maximum diameter of 20 mm were sacrificed. Resulting tumors and organs were examined at necropsy and harvested for further experimentation. Tumor multiplicity was calculated as the average number of tumors per animal visualized macroscopically at necropsy.

Orthotopic tumor assay. For mouse-derived cell lines, 2.5×10^5 cells were injected into the inguinal mammary fat pad of *nu/nu* mice. For human-derived cell lines, 4×10^6 cells were injected into the inguinal mammary fat pad of *nu/nu* mice. Animals were monitored for tumor formation every 2 days. Once tumors were detected, measurements were obtained twice weekly. Mean tumor growth and tumor volume was calculated as previously described (94, 95). Mice possessing tumors with a maximum diameter of 20 mm were sacrificed and tumors harvested for analysis.

Retrovirus production and infection. Oligonucleotides for shRNAs targeting Hunk were designed using RNAi central (http://katahdin.cshl.org/siRNA/RNAi.cgi?type=shRNA). The following sequences were used: Hunk shRNA 1: TGCTGTTGACAGTGAGCGCAGAACCTGCCTTCTCA CAAACTAGTGAAGCCACAGATGTAGTTTGTGAGAAGGCAGGTTCTTTGCCTACTGCCTCGGA; Hunk shRNA 2: TGCTGTTGACAGTGAGCGCTGCTACTAGATGAAGACAATATAGTGAAGCCACAGATGTATATTGTCTTCATCTAGTAGCAATGCCTACTGCCTCGGA; HUNK shRNA: TGCTGTTGACAGTGAGCGAATAGAGAATTTGCTACTAGATTAGTGAAGCCACAGATGTAATCTAGTAGCAAATTCTCTATCTGCCTACTGCCTCGGA.

Oligonucleotides were cloned into pGIPZ or LMP vectors (a gift from S. Lowe, Cold Spring Harbor Laboratory, New York, USA) as previously described (96). Retroviral vectors expressing wild-type Hunk or Hunk K91M and retroviral supernatants were generated as described (14). HER2 (Addgene plasmid 16257) was subcloned to the PK1 retroviral vector (a gift from Warren Pear, University of Pennsylvania, Philadelphia, Pennsylvania, USA).

Immunoblotting and immunoprecipitation. Protein lysates were prepared by homogenizing tumors or cell lines in lysis buffer (50 mM Tris-HCl, pH 7.9; 150 mM NaCl; 1% Triton X-100) supplemented with 1 tablet of Complete protease inhibitors (Roche) per 20 ml buffer, 1 mM glycerol β-phosphate, and 0.1 M NaF. For immunoprecipitation assays, cell lysates were incubated overnight at 4°C with control IgG, HER2 antibody, or Hunk antibody bound to Protein A/G Sepharose mix. Beads were washed 4 times with lysis buffer. For Western blot analysis, membranes were probed with peroxidase-conjugated secondary antibodies (Jackson Laboratories) or Alexa-Fluor antibody (Molecular Probes). Bound antibodies were detected with an enhanced chemiluminescent system (ECL; Amersham) or using the Odyssey detection system (LI-COR Biosciences). The following primary antibodies were used during Western blotting: anti-Hunk (14); anti-ErbB2 (Cell Signaling); anti–β-tubulin (Biogenex); anti–phospho-Akt and total Akt (Cell Signaling), anti–phospho-Erk1/2 and total Erk1/2 (Cell Signaling), and anti-p27 (Cell Signaling).

RNA isolation and QRT-PCR. RNA from tumors was prepared by homogenization of snap-frozen tissue samples or tissue culture cells in guanidinium isothiocyanate supplemented with 7 μl/ml 2-mercaptoethanol followed by ultracentrifugation through cesium chloride as described (97, 98). Poly$(A)^+$ RNA was selected using oligo(dT) cellulose (Pharmacia), separated on a 0.7% LE agarose gel, and passively transferred to a Gene Screen membrane (NEN). Northern hybridization was performed as described using a ^{32}P-labeled cDNA probe encompassing nucleotides 1,149 to 3,849 of *Hunk* generated by random-primed labeling (Roche) (98). Hybridization was performed at a concentration of 10^6 cpm/ml in 48% formamide, 10% dextran sulfate, 4.8× SSC, 20 mM Tris (pH 7.5), 1× Denhart's solution, 20 μg/ml salmon sperm DNA, and 0.1% SDS at 42°C overnight. Following hybridization, blots were washed twice in 2× SSC/0.1% SDS at room temperature for 30 minutes, followed by 2 washes in 0.2× SSC/0.1% SDS at 50°C for 20 minutes, and subject to autoradiography (Kodak XAR-5).

RNA from cells was isolated using RNeasy RNA isolation kit (Qiagen) according to the manufacturer's protocol. Reverse transcription was performed using the Advantage cDNA High Capacity Reverse Transcriptase Kit (Applied Biosystems) according to the manufacturer's protocol. Briefly, 2 μg of total RNA were incubated with the provided buffers consisting of dNTPs, random primers, RT, and RNase inhibitor and subjected to incubation at 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 seconds. The resulting cDNA was used to perform QRT-PCR on the Applied Biosystems 7900 HT Fast Real-Time PCR system using 6-carboxyfluorescein–labeled Taqman probes (Applied Biosystems) specific for Hunk, neu, and TBP.

Immunofluorescence. Immunofluorescence staining of paraffin sections was performed by preparing tumor sections using a standard xylene-based de-waxing procedure. Sections were subjected to antigen retrieval in the 2100 Retriever (Electron Microscopy Sciences) prior to blocking in 3% BSA, 10% normal

goat serum, and PBS. Primary antibodies were incubated in blocking buffer overnight at 4°C with shaking. Secondary Alexa Fluor–conjugated (Molecular Probes) antibodies were incubated in 3% BSA and PBS, followed by Hoechst staining to visualize nuclei. Primary antibodies consisted of anti-Ki67 (Leica), anti–cleaved caspase-3 (Cell Signaling), and anti-p27 (BD Biosciences).

Acknowledgments

This work was supported by grants from the National Cancer Institute, including R01 CA127917, R01 CA098371, UO1 CA105490, and F32 CA130313 (to E.S. Yeh); and from the Department of Defense Breast Cancer Research Program, including W81XWH-05-1-0405 and W81XWH-08-1-0307 (to E.S. Yeh), as well as other NCI, DOD, and foundation research grants.

Received for publication March 10, 2010, and accepted in revised form December 29, 2010.

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