The Snf1-related kinase, Hunk, is essential for mammary tumor metastasis

Gerald B. W. Wertheim[a], Thomas W. Yang[a], Tien-chi Pan[a], Anna Ramne[a], Zhandong Liu[a], Heather P. Gardner[a], Katherine D. Dugan[a], Petra Kristel[b], Bas Kreike[b], Marc J. van de Vijver[b], Robert D. Cardiff[c], Carol Reynolds[d], and Lewis A. Chodosh[a,1]

[a]Departments of Cancer Biology, Cell and Developmental Biology, and Medicine, Abramson Family Cancer Research Institute, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6160; [b]Department of Diagnostic Oncology, The Netherlands Cancer Institute, Antoni van Leeuwenhoek Hospital, Plesmanlaan 121, 1066 CX, Amsterdam, The Netherlands; [c]Center for Comparative Medicine, University of California, County Road 98 and Hutchison Drive, Davis, CA 95616; and [d]Division of Anatomic Pathology, Mayo Clinic, Rochester, MN 55905

Communicated by Craig B. Thompson, University of Pennsylvania, Philadelphia, PA, July 27, 2009 (received for review April 22, 2009)

We previously identified a SNF1/AMPK-related protein kinase, Hunk, from a mammary tumor arising in an MMTV-neu transgenic mouse. The function of this kinase is unknown. Using targeted deletion in mice, we now demonstrate that Hunk is required for the metastasis of c-myc-induced mammary tumors, but is dispensable for normal development. Reconstitution experiments revealed 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. Consistent with a role for this kinase in the progression of human cancers, the human homologue of Hunk is overexpressed in aggressive subsets of carcinomas of the ovary, colon, and breast. In addition, a murine gene expression signature that distinguishes Hunk-wild type from Hunk-deficient mammary tumors predicts clinical outcome in women with breast cancer in a manner consistent with the pro-metastatic function of Hunk in mice. These findings identify a direct role for Hunk kinase activity in metastasis and establish an in vivo function for this kinase.

breast cancer | knockout mice | mouse models | protein kinase

Despite continuing advances in prevention and treatment, breast cancer remains the leading cause of cancer mortality among women worldwide (1). The primary determinant of mortality in breast cancer patients is the metastatic spread of tumor cells to distant sites (2). Although metastases have classically been thought to arise from rare cells within a primary tumor (3), microarray expression profiling experiments suggest that the propensity for tumors to metastasize may be established early in tumor development and therefore may be shared by many cells (4). As such, identifying genetic pathways that determine metastatic potential in primary tumors is a critical priority in cancer research.

We have identified a 80-kDa serine/threonine kinase, Hunk/Mak-V/Bstk1 (*H*ormonally *u*pregulated *n*eu-associated *k*inase), in an epithelial cell line established from a mammary carcinoma arising in an MMTV-neu transgenic mouse (5–8). The catalytic domain of Hunk established it as a member of the SNF1/AMP-activated protein kinase (AMPK) family. Hunk expression in the mouse is restricted to subsets of epithelial cells within multiple tissues, including the mammary gland, and is up-regulated during early pregnancy and in response to 17β-estradiol and progesterone (5–7). The function of this kinase, however, is unknown.

We now describe the generation and characterization of Hunk-deficient mice and demonstrate that Hunk kinase activity is required for mammary tumor cells to gain access to the circulation and metastasize. Consistent with its pro-metastatic function in mice, we find that the human homologue of Hunk is overexpressed in histopathologically advanced subsets of human cancers. Furthermore, using microarray expression profiling, we demonstrate that a set of genes that distinguishes Hunk-wild type from Hunk-deficient mouse tumors predicts metastasis-free survival in women with breast cancer. Together, our findings establish a role for Hunk in tumor progression and identify this kinase as a target for anti-neoplastic therapy.

Results

Hunk Is Overexpressed in Aggressive Subsets of Human Cancers. To investigate its role in human tumorigenesis, we cloned the human homologue of Hunk from a fetal brain cDNA library. Sequence analysis yielded a composite cDNA spanning an ORF of 714 amino acids (GenBank accession #NM_014586). Review of this sequence and of human genome data indicated that a single Hunk isoform exists that is 92% identical to murine Hunk at the amino acid level (Fig. S1). RNase protection analysis demonstrated that Hunk exhibits a wide range of expression in human breast, colon, and ovarian cancer cell lines (Fig. 1*A*).

Consistent with the wide range of Hunk expression in human cancer cell lines, analysis of Hunk mRNA levels in human primary breast cancers revealed an approximate 160-fold range of expression with 39% of human breast cancers (60/153) expressing Hunk at levels three standard deviations (1.4-fold) greater than the mean observed in normal breast tissue (Fig. 1*B*). Conversely, 24% of tumors (36/153) expressed Hunk at levels three standard deviations (two-fold) lower than the mean of normal breast tissue. Notably, Hunk expression was significantly higher in lymph node-positive breast cancers compared to node-negative cancers ($P = 2.9 \times 10^{-5}$), and in HER2/neu-expressing compared to non-expressing breast cancers ($P = 0.01$). Thus, Hunk is overexpressed in human breast cancers bearing at least two pathologic hallmarks of aggressive disease. In an analogous manner, human primary cancers of the colon and ovary also exhibited a wide range of Hunk expression with higher expression observed in more poorly differentiated carcinomas (Fig. 1 *C* and *D*).

Hunk Is Dispensable for Murine Development. The correlation between Hunk expression and markers of aggressive disease in human cancers led us to assess the tumorigenic role of Hunk in vivo. To this end, we generated mice bearing a targeted deletion in the *Hunk* locus (Fig. 2*A* and Fig. S2). Crosses between $Hunk^{\Delta 1/+}$ mice gave rise to offspring in Mendelian ratios. Immunoprecipitation of Hunk from lung extracts—a site of high Hunk expression—followed by immunoblotting with a Hunk-specific polyclonal antibody revealed that $Hunk^{\Delta 1/\Delta 1}$ mice do not express detectable Hunk protein, confirming that this mutation generates a null allele (Fig. 2*B*).

$Hunk^{\Delta 1/\Delta 1}$ mice exhibited similar viability, fertility, and longevity compared to controls and did not display alterations in organogenesis or a propensity to develop spontaneous tumors. Moreover, analysis of mammary gland development failed to reveal morpho-

Author contributions: G.B.W.W., T.W.Y., H.P.G., and L.A.C. designed research; G.B.W.W., T.W.Y., A.R., H.P.G., K.D.D., R.D.C., and C.R. performed research; P.K., B.K., and M.J.v.d.V. contributed new reagents/analytic tools; G.B.W.W., T.W.Y., T.-c.P., A.R., Z.L., H.P.G., R.D.C., C.R., and L.A.C. analyzed data; and G.B.W.W., T.W.Y., T.-c.P., and L.A.C. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

[1]To whom correspondence should be addressed. E-mail: chodosh@mail.med.upenn.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0906993106/DCSupplemental.

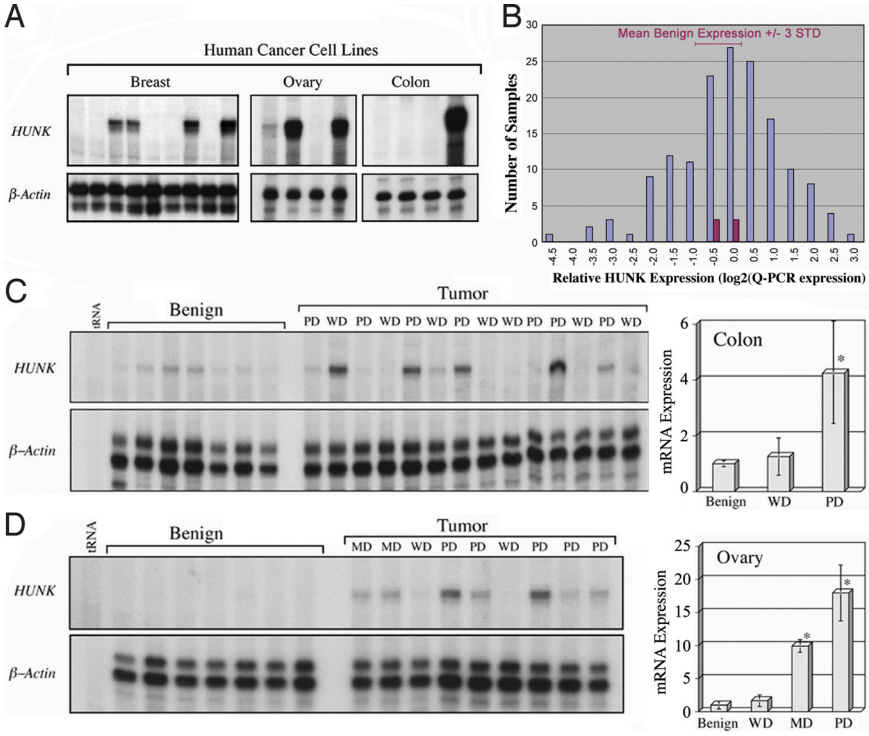

Fig. 1. Hunk is overexpressed in aggressive subsets of human cancers. (*A*) RNase protection analysis of Hunk and β-actin expression in a panel of human breast, ovarian, and colon cancer cell lines. (*B*) Histogram of relative *Hunk* expression normalized to *TBP* in a panel of primary human breast cancers and normal human breast samples determined by quantitative RT-PCR. Expression levels are displayed as log2 and are relative to mean expression in normal breast tissue. The range of Hunk expression falling within three standard deviations of the mean for normal breast tissue is indicated. (*C* and *D*) RNase protection analysis of *Hunk* expression in a representative panel of human primary colon (*C*) and ovarian (*D*) carcinomas and corresponding normal tissues. WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated. Hunk expression was significantly elevated in poorly differentiated colon cancers compared to well-differentiated cancers or benign colon tissue ($P = 0.028$ and $P = 0.0036$, respectively) and in moderately to poorly differentiated ovarian cancers compared to well differentiated ovarian cancers ($P = 2.0 \times 10^{-6}$).

logic or functional differences among Hunk genotypes (Fig. S3). Thus, Hunk is dispensable for murine development, including that of the mammary gland.

Hunk Is Required for Mammary Tumor Metastasis. To determine whether Hunk is required for mammary tumorigenesis, Hunk-deficient mice were crossed to MMTV-*c-myc* transgenic mice that constitutively express the c-myc oncoprotein throughout the mammary epithelium (9). *MYC* is amplified in 10–30% of human breast cancers, is overexpressed in approximately 50% of human breast cancers, and is associated with poor prognosis (10–12). No differences in tumor latency, multiplicity, or growth rates were observed between Hunk wild type, heterozygous, or homozygous MMTV-c-myc mice (Fig. 2*C*). Furthermore, c-myc-induced tumors in Hunk-wild type and Hunk-deficient mice were histologically indistinguishable (Fig. 2*D*). These data indicate that Hunk is not required for myc-induced mammary tumorigenesis.

At necropsy, a subset of animals was found to have gross lung nodules that were determined to be metastases by histological analysis (Fig. 3 *A* and *B*). Notably, the fraction of mice with lung metastases was nearly five-fold lower for Hunk-deficient mice compared to Hunk-wild type mice (Fig. 3*C*, $P < 0.0001$). Hunk-heterozygous mice exhibited an intermediate metastatic rate that was significantly higher than that observed in homozygous Hunk-deficient mice (Fig. 3*C*, $P = 0.02$). These findings demonstrate that Hunk is required for efficient metastasis of myc-induced mammary tumors.

Hunk-Deficient Tumor Cells Fail To Escape the Mammary Fat Pad. Successful metastasis requires tumor cells to detach from neighboring cells, invade adjacent tissue, intravasate into the vasculature or lymphatic system, survive in the circulation, arrest on vessel walls, extravasate from the circulation into distant organs, and proliferate at distant sites (3). The efficiency with which each of these steps of the metastatic cascade occurs is influenced by tumor cell characteristics and the host environment. As such, the metastatic defect observed in Hunk-deficient mice could be due to the absence of Hunk in tumor cells or in other cell types, including those of the mammary stroma, vasculature, or lung.

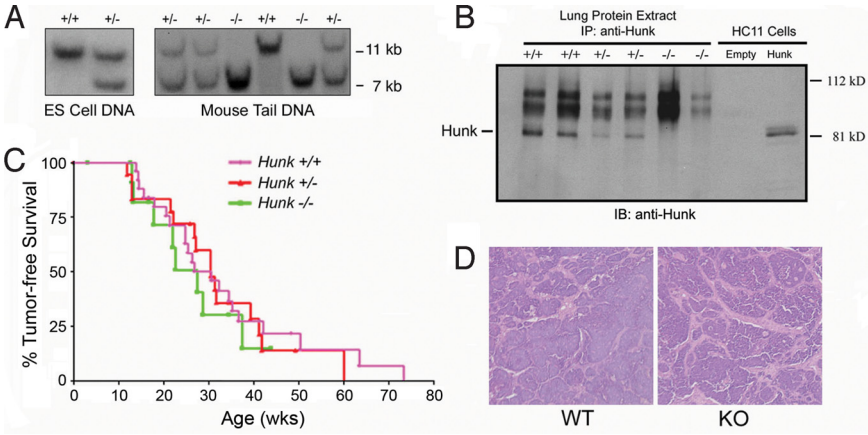

Fig. 2. Myc-induced primary mammary tumorigenesis is unaffected in Hunk-deficient mice. (*A*) Southern hybridization analysis demonstrating generation of mice heterozygous and homozygous for targeted deletion of Hunk. Genomic DNA was digested with *Xmn* I and subjected to Southern hybridization using a Hunk 3′ probe. (*B*) Hunk protein expression in Hunk wild type (+/+), heterozygous (+/−), and homozygous mutant (−/−) mice. Protein lysates from lung tissue of mice of the indicated Hunk genotypes were immunoprecipitated and immunoblotted with anti-Hunk C-terminal specific antiserum. Controls consisting of protein lysates from HC11 cells transfected with an empty vector or a Hunk-expression vector are shown. Ig bands are also identified in the first six lanes. (*C*) Tumor-free survival curves for MMTV-c-myc mice wild type ($n = 28$), heterozygous ($n = 18$), or homozygous ($n = 16$) for null mutations in Hunk. No statistical difference among the three Hunk genotypes was observed. (*D*) Histology of Hunk-wild type and Hunk-deficient MMTV-c-myc-induced mammary tumors.

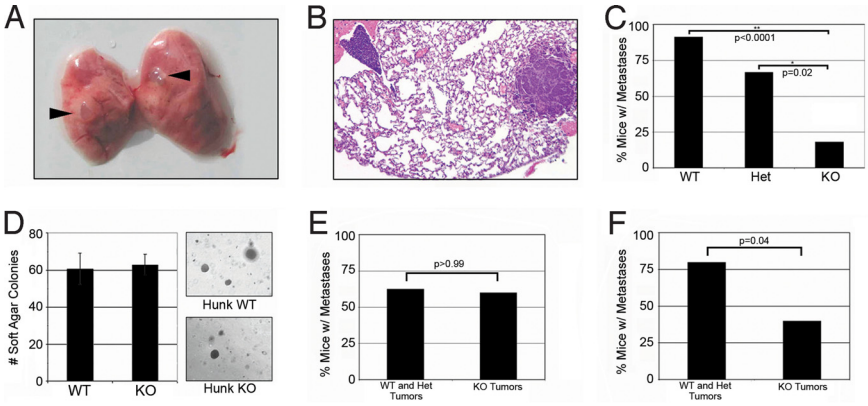

Fig. 3. Hunk-deficient mice display a tumor cell-instrinsic defect in metastasis. Gross (*A*) and histological (*B*) appearance of lung metastases arising from mammary tumors in MMTV-*c-myc*-mice. (*C*) Percentage of mice with grossly visible lung metastases within each Hunk genotype. Significantly fewer Hunk-deficient mice ($n = 11$) harbored tumor metastases compared to Hunk wild type ($n = 23$) or heterozygous ($n = 15$) controls (Fisher's exact test). (*D*) Soft agar colony formation assay for Hunk-wild type and Hunk-deficient primary tumor cells from MMTV-c-myc mice. (*E*) Metastatic frequency following tail vein injection of primary tumor cells into the circulation of nude mice. (*F*) Metastatic frequency following orthotopic injection of primary tumor cells into the fat pads of nude mice. A significantly lower frequency of metastasis was observed for Hunk-deficient mammary tumor cells ($P = 0.04$).

To identify steps in the metastatic cascade that are influenced by Hunk, we first performed soft-agar colony assays. No differences were observed in the number or size of colonies formed by Hunk-wild type and Hunk-deficient tumor cells (Fig. 3*D*), suggesting that Hunk is not required for tumor cells to evade anoikis. To extend these findings, primary tumor cells were introduced into the circulation of *nu/nu* mice via tail vein injection. Consistent with results in soft agar, Hunk-wild type and Hunk-deficient tumor cells displayed a similar ability to form metastatic lung nodules (Fig. 3*E*). In contrast, primary tumor cells orthotopically transplanted into the mammary fat pads of *nu/nu* mice recapitulated the reduced metastatic potential observed in Hunk-deficient tumors (Fig. 3*F*). These results demonstrate that the metastatic defect observed in Hunk-deficient tumors is due, at least in part, to the absence of Hunk in tumor cells rather than the surrounding stroma. Furthermore, this defect appears to result from a decreased ability of Hunk-deficient tumor cells to escape the mammary fat pad, and not subsequent steps.

To confirm these results, we established cell lines from MMTV-c-myc tumors that were either Hunk-wild type (MW1 and MW4) or Hunk-deficient (MK1). To determine whether a Hunk-dependent difference in metastatic behavior was retained in these cell lines, cohorts of *nu/nu* mice were orthotopically injected with tumor cells from each line. Mice were killed when tumors reached a mean cross-sectional area of 400 mm^2. Although growth rates of tumors were comparable, mice bearing MK1-derived tumors exhibited a 3.2-fold decreased incidence of metastasis compared to animals bearing MW1- or MW4-derived tumors (Fig. 4*A*; $P = 0.0003$ and $P = 0.0004$, respectively). When animals were killed with smaller tumors (cross-sectional area 225 mm^2), 75% (6/8) of mice harboring MW1 orthotopic tumors had visible metastases, whereas none of the mice harboring MK1-derived tumors exhibited metastases (0/8) (Fig. 4*B*; $P = 0.007$). These data confirm a tumor cell-instrinsic requirement for Hunk for the efficient metastasis of myc-induced tumors.

Hunk Directly Influences Metastatic Behavior. Our finding that Hunk is required for the metastasis of myc-induced tumors did not distinguish whether Hunk directly regulates cellular processes essential for metastasis or is instead required for the establishment of a tumor cell type predisposed to metastasis. To address this, we orthotopically transplanted Hunk-deficient MK1 tumor cells that were retrovirally transduced with either a control vector (MK1E) or a vector expressing wild-type Hunk (MK1H) into the fat pads of *nu/nu* mice. Mice were killed when tumors reached a cross-sectional area of 225 mm^2. As with primary tumors, no differences were

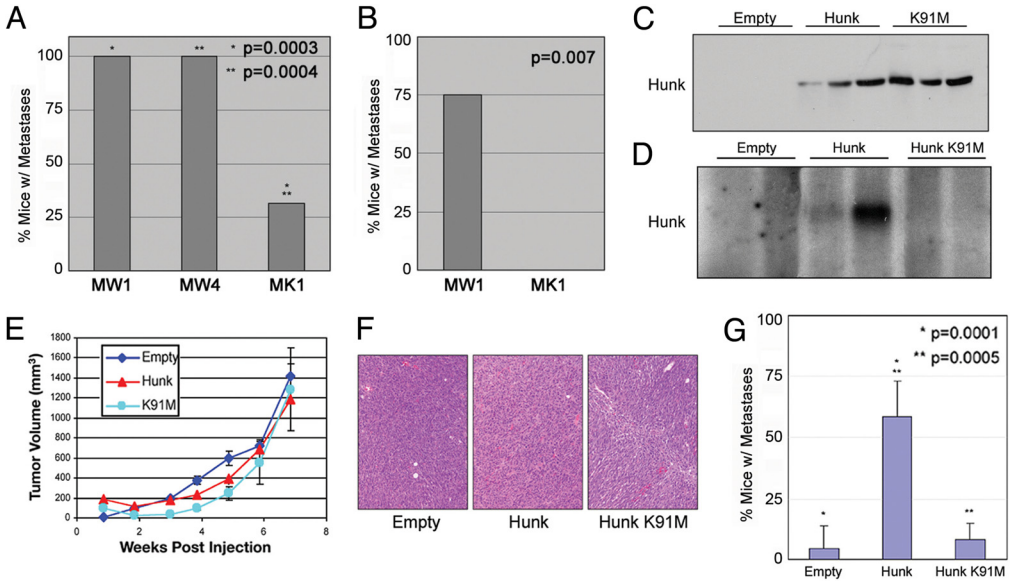

Fig. 4. Hunk kinase activity is required for efficient metastasis of myc-induced mammary tumors. (*A*) Percentage of mice injected with primary tumor cell lines with grossly visible lung metastases. Mice injected with Hunk-wild type cell lines (MW1, $n = 11$; MW4, $n = 10$) and Hunk-deficient cell lines (MK1, $n = 19$) were killed when tumors reached a mean cross-sectional area of 400 mm^2. Significantly fewer lung metastases were observed in mice bearing MK1-derived tumors (Fisher's exact test). (*B*) Percentage of mice with grossly visible lung metastases after injection with MW1 ($n = 8$) or MK1 ($n = 8$) cell lines and killed when mean cross-sectional tumor area reached 225 mm^2. (*C* and *D*) Western blot (*C*) and in vitro kinase assay (*D*) of MK1 cells stably transduced with an empty vector, a Hunk-expression vector, or a Hunk K91M-expression vector. Autophosphorylation of Hunk is specifically detected in wild-type Hunk expressing cells. (*E* and *F*) Tumor growth rates (*E*) and histologic appearance (*F*) of tumors derived from MK1 cell lines transduced with an empty vector (blue), wild type Hunk-expression vector (red), or a Hunk-K91M expression vector (teal). (*G*) Percentage of mice injected with primary tumor cell lines with grossly visible lung metastases. Mice were killed when tumors reached a mean cross-sectional area of 225 mm^2. Significantly more lung metastases were observed in mice bearing tumors derived from Hunk-expressing cell lines ($n = 24$) compared to vector control cell lines ($n = 22$) or cell lines expressing a Hunk-K91M allele ($n = 24$) (Fisher's exact test).

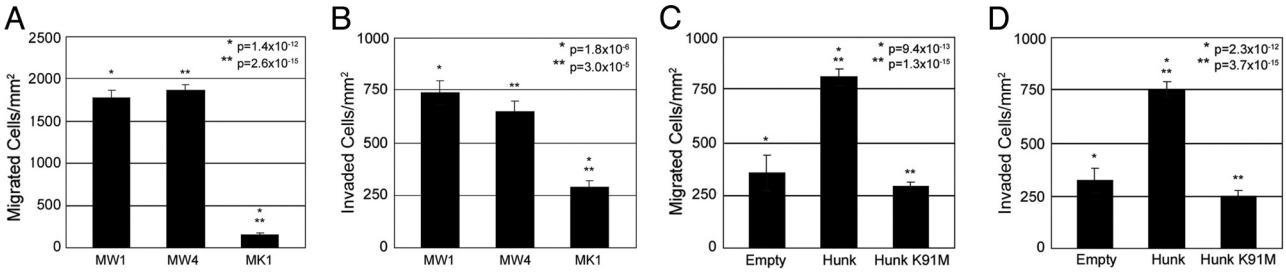

Fig. 5. Myc tumor cell invasion and migration require Hunk kinase activity. (*A–D*) Number of cells per mm^2 translocated in uncoated migration chambers (*A* and *C*) or matrigel coated migration chambers (*B* and *D*). Cells were seeded in migration chambers and allowed to translocate for 18 h. (*A* and *B*) A significantly lower number of Hunk-deficient tumor cells (MK1) translocated relative to Hunk-wild type tumor cells (MW1, MW4). (*C* and *D*) A significantly higher number of wild type Hunk-transduced MK1 cells translocated relative to cells transduced with empty vector or cells transduced with Hunk-K91M.

observed in the growth rates or histology of orthotopic tumors (Fig. 4 *E* and *F*). However, examination of the lungs of mice bearing tumors derived from Hunk-expressing MK1H cells revealed an 11.6-fold increased incidence in metastasis compared to empty vector MK1E controls (Fig. 4*G*; $P = 0.0001$). This indicates that Hunk directly modulates metastatic behavior.

Hunk Kinase Activity Is Required for Metastasis. To determine whether the requirement for Hunk in metastasis is dependent upon kinase activity, we generated a kinase-dead allele of Hunk (Hunk-K91M). Stably transduced MK1 cells were generated that expressed comparable levels of wild-type Hunk (MK1H) or Hunk-K91M (MK1K) relative to empty vector controls (MK1E) (Fig. 4*C*). Expression of wild type Hunk, but not Hunk-K91M, resulted in an increase in kinase activity immunoprecipitated by anti-Hunk polyclonal antibodies (Fig. 4*D*), confirming that the K91M substitution results in an inactive kinase.

To determine the ability of the K91M allele to rescue the metastatic defect of Hunk-deficient tumor cells, we orthotopically transplanted MK1 tumor cells retrovirally transduced with Hunk-K91M (MK1K) into the fat pads of *nu/nu* mice. Growth rates and histologic appearance of MK1K-derived tumors were similar to those of MK1E and MK1H-derived tumors (Fig. 4 *E* and *F*). The incidence of lung metastases from MH1K-derived tumors was 7.3-fold lower than that observed with MK1H-derived tumors (Fig. 4*G*; $P = 0.0005$) and was comparable to that observed with MK1E-derived tumors. Thus, the ability of Hunk to promote mammary tumor metastasis requires its kinase activity.

Hunk Is Required for Tumor Cell Migration and Invasion. We next sought to determine whether the metastatic defect observed in Hunk-deficient tumor cells could be attributed to decreased migratory or invasive properties. MW1, MW4, and MK1 cells were seeded into uncoated migration chambers and cells were counted that translocated to the bottom of the lower chamber within 18 h. In multiple experiments, >10-fold-fewer Hunk-deficient MK1 cells migrated across the uncoated membrane compared to Hunk-wild type MW1 and MW4 cell lines (Fig. 5*A*; $P = 1.4 \times 10^{-12}$ and $P = 2.6 \times 10^{-15}$, respectively). MW1, MW4, and MK1 cells were then seeded in Matrigel-coated migration chambers. Similar to results obtained with uncoated chambers, Hunk-deficient MK1 cells translocated less frequently (≈2.4-fold) than their wild-type counterparts (Fig. 5*B*; $P = 1.8 \times 10^{-6}$ and $P = 3.0 \times 10^{-5}$, respectively).

We then assayed the migration and invasive properties of Hunk-deficient MK1 cells stably transduced with wild-type Hunk, Hunk-K91M, or an empty vector control. After seeding in uncoated migration chambers, Hunk-expressing MK1H cells translocated approximately 2.3-fold more frequently than MK1E cells and 2.8-fold more frequently than MK1K cells (Fig. 5*C*; $P = 9.4 \times 10^{-13}$ and $P = 1.3 \times 10^{-15}$, respectively). Similarly, when plated on Matrigel-coated migration chambers, MK1H cells translocated approximately 2.3-fold more frequently than MK1E cells and ≈3.0-fold more frequently than MK1K cells (Fig. 5*D*; $P = 2.3 \times 10^{-12}$ and $P = 3.7 \times 10^{-15}$, respectively). These results demonstrate that Hunk kinase activity is required for the migration and invasion of c-myc induced mammary tumor cells.

A Hunk-Associated Expression Profile Predicts Metastasis-Free Survival in Women with Breast Cancer. Previous studies have demonstrated that the propensity of breast cancers to metastasize is often reflected in the gene expression profile of the primary tumor (4, 13–17). Therefore, the distinct metastatic behavior of Hunk-wild type and Hunk-deficient mammary tumors suggested that reproducible differences might exist in their gene expression profiles. To test this, we compared the gene expression profiles of c-myc-induced mammary tumors from Hunk-wild type and Hunk-deficient mice using Affymetrix MOE430A oligonucleotide arrays. We identified 110 genes differentially expressed between Hunk-wild type and Hunk-deficient mice (Fig. 6*A* and Table S1). Leave-one-out cross validation performed on these tumors using a nearest-centroid approach achieved a prediction accuracy of 100%. Thus, primary tumors in Hunk-wild type and Hunk-deficient mice are molecularly distinct.

Since Hunk is required for the efficient metastasis of myc-induced mammary tumors in mice, and since Hunk expression in human cancers is associated with aggressive phenotypes, we hypothesized that our Hunk-deficient mouse tumor model might recapitulate clinically relevant aspects of human tumor progression. Therefore, we used the set of 110 genes in a nearest-centroid approach to classify human breast cancers in multiple data sets (14, 15, 18, 19).

As predicted, women with breast cancers bearing expression profiles similar to Hunk-wild type tumors had an increased risk of metastasis (15) ($P = 0.027$), recurrence (19) ($P = 0.026$), distal recurrence (19) ($P = 0.0032$), regional recurrence (18) ($P = 0.035$), and death (18) ($P = 0.001$) (14) ($P = 0.039$) compared to women with breast cancers bearing gene expression profiles similar to Hunk-deficient tumors (Fig. 6). Furthermore, breast cancers classified as similar to Hunk-wild type tumors possessed histopathological characteristics associated with poor prognosis, including ER-negativity ($P = 1.5 \times 10^{-5}$, 7.8×10^{-6}, and 1.2×10^{-2}) (15, 18, 19), basal-like subtype ($P = 2.8 \times 10^{-6}$, 1.4×10^{-4}, and 4.9×10^{-3}), and advanced tumor grade ($P = 1.6 \times 10^{-2}$, 3.1×10^{-7}, and 1.4×10^{-3}) (15, 18, 19). These results suggest that gene expression differences distinguishing Hunk-wild type from Hunk-deficient mammary tumors in mice reflect molecular differences in human breast cancer that are relevant to metastatic potential and prognosis.

Discussion

Recent successes in the clinical application of kinase inhibitors for the treatment of human cancers have highlighted the importance of kinases in carcinogenesis and have generated intense interest in the development of targeted therapeutics against this class of molecules (20, 21). As breast cancer is the most common malignancy among

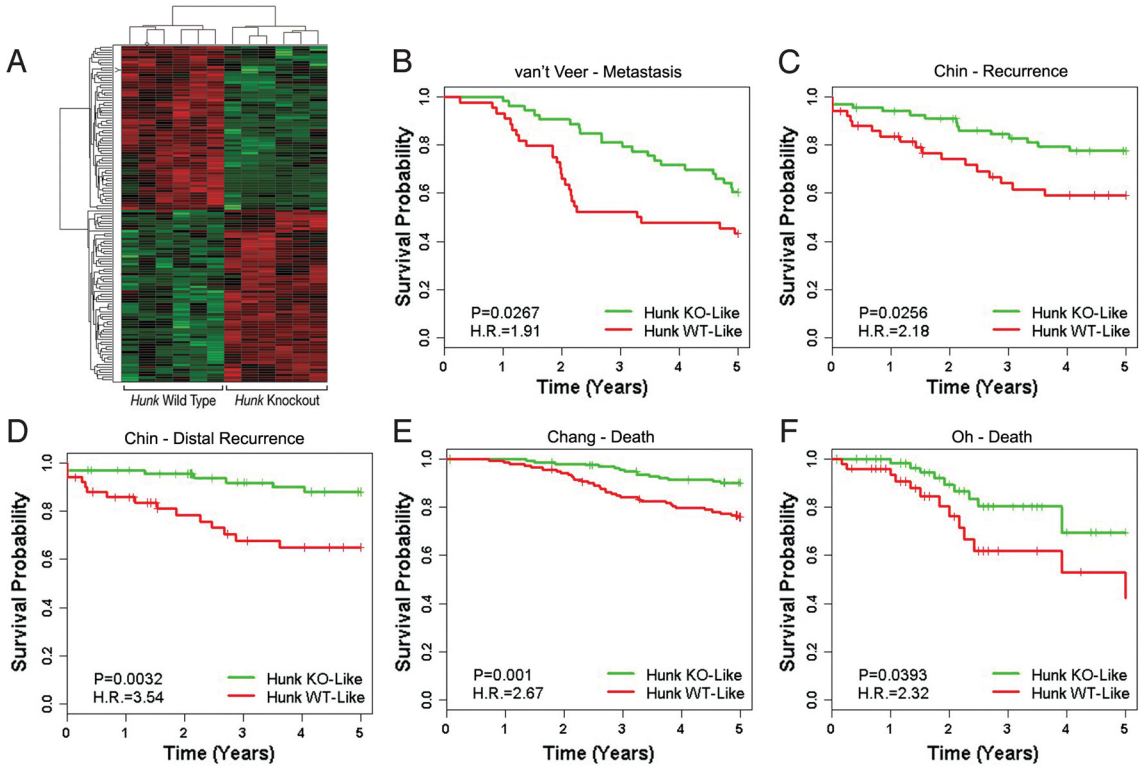

Fig. 6. A mouse *Hunk*-expression centroid predicts metastasis-free survival in women with breast cancer. (*A*) Supervised hierarchical clustering of Hunk-wild type and Hunk-deficient mouse mammary tumors based on genes differentially expressed between the two groups at a false discovery rate of 0.1%. (*B–F*) Survival curves for breast cancers in the data sets of (14, 15, 18, 19). Cancers were classified as similar to Hunk-wild type or Hunk-deficient murine tumors based upon the similarity of their gene expression profiles to an expression centroid distinguishing Hunk-wild type from Hunk-deficient MMTV-*c-myc* mammary tumors. Log rank test *p*-values and hazard ratios are shown.

women worldwide and the leading cause of cancer mortality, identifying protein kinases that contribute to the development and progression of this malignancy is a critical priority.

In this study, we have established a function for the protein kinase Hunk by deleting it from the mouse germline. Evaluation of Hunk-deficient mice revealed that Hunk is dispensable for normal development, but is required for efficient metastasis of myc-induced mammary tumors, as well as for tumor cell migration and invasion. These defects occurred in the absence of effects on the latency, multiplicity, or growth of primary tumors.

Notably, the requirements for Hunk in tumor cell migration, invasion, and metastasis were direct as re-expression of Hunk in Hunk-deficient tumor cells rescued defects in each of these properties. Moreover, the ability of Hunk to promote migration, invasion, and metastasis was dependent upon its kinase activity, suggesting that these phenotypes are mediated by substrates of Hunk. Together, these findings constitute a demonstration of an in vivo function for Hunk and establish it as one of relatively few proteins that have been shown to be required for metastasis of spontaneous tumors in intact animal models.

Hunk is a member of the Snf1/AMPK family of kinases. The yeast Snf1 kinase and its mammalian homologue, AMPK, are activated by reduced energy availability and are required for physiological responses to nutrient starvation (22). AMPK family members have also been implicated in the response to DNA-damage, cell proliferation, cell survival, and oncogenic signaling (23–27). A number of Snf1/AMPK-related kinases affect cellular responses to stress, cell migration and invasion. For example, Ark5 promotes cell survival in response to nutrient deprivation and death receptor signaling and is up-regulated during colorectal cancer progression, with highest levels of expression observed in metastases (28–30). Consistent with this, enforced expression of Ark5 enhances the metastatic potential of pancreatic tumor cell lines by increasing cellular migration and invasion (29, 31).

Similarly SNARK promotes cellular migration in mammalian cells deprived of glucose (32). Snf1 itself plays a role in invasion as yeast grown on non-fermentable carbon sources exhibit Snf1-dependent invasive growth (33, 34). Thus, multiple Snf1/AMPK family members, including Hunk, may play roles in mediating invasive cellular responses to nutrient deprivation.

We have demonstrated that Hunk is overexpressed in aggressive subsets of human carcinomas. Consistent with a role for Hunk in human tumor progression, a murine gene expression classifier distinguishing Hunk-wild type from Hunk-deficient mammary tumors predicted metastasis-free and recurrence-free survival in women with breast cancer in multiple patient data sets. Furthermore, human breast cancers identified as similar to Hunk-wild type tumors in mice displayed histopathologic hallmarks of aggressive disease. Together, our findings suggest that Hunk may play a role in the aggressive behavior of human cancers, including metastasis of human breast cancers.

Finally, our finding in mice that Hunk is dispensable for normal development suggests that inhibition of this kinase may lack significant deleterious consequences. This finding, together with our observations that Hunk kinase activity is required for mammary tumor metastasis, and that Hunk expression is associated with aggressive subsets of human cancers, identify Hunk as a potential target for anti-neoplastic therapy.

Experimental Procedures

Animal and Tissue Preparation. Mice were housed under barrier conditions with a 12-h light/dark cycle. For histological analysis, tumors were fixed in 4% paraformaldehyde overnight and transferred to 70% ethanol before paraffin embedding; sections were cut and stained with hematoxylin and eosin.

Cloning of Human *Hunk* cDNA. A probe containing nucleotides 276 to 793 of *Hunk* (GenBank Accession #AF167987) was used to screen a fetal brain cDNA library (Stratagene) as described (5). Two overlapping clones spanning the entire ORF were sequenced on both strands to obtain the composite nucleotide and amino acid sequence.

Analysis of Hunk Expression. RNA was prepared as described (35). Nucleotides 359 to 582 of human *Hunk* or nucleotides 1,142 to 1,241 of *ß-actin* (GenBank accession # X03672) were used as probes for RNase protection analysis (35). For quantitative RT-PCR, cDNA was generated from total RNA using the SuperScript First-strand synthesis system (Invitrogen). For details of cell lines and RT-PCR primers and probes, see *SI Text*.

Generation and Analysis of Hunk-Deficient Mice. A 129/Sv mouse genomic library (Stratagene) was screened with a *Hunk* cDNA fragment (nt 1–706) (5). The *Hunk* gene was disrupted by replacement of a 1.1-kb fragment containing the putative promoter and exon 1 of *Hunk* with a pGK*neo* cassette flanked by *Lox*P sites. Linearized vector was electroporated into E14 ES cells and selected in 300 μg/mL G418. Properly targeted clones were identified by Southern hybridization using a 3′ *Xho*I/*Xmn* I flanking probe. Lung protein lysates were subjected to immunoprecipitation and immunoblotting with Hunk-specific C-terminal antisera as described (6).

Tumorigenesis and Metastasis Assays. Hunk-deficient mice were crossed to MMTV-c-myc mice (36). MMTV-c-myc female animals of each *Hunk* genotype were mated twice, then monitored twice weekly for mammary tumors. Mice possessing tumors with a maximum diameter of 20 mm were killed, tumors harvested and organs examined at necropsy (see *SI Text*). Tumor nodules were identified by examination of organs through a Leica Wild MZ8 dissection microscope.

For tail vein injections, 5×10^5 cells were injected and mice were killed eight weeks postinjection. For orthotopic injection, 5×10^6 cells were injected into the left #4 mammary fat pad of anesthetized (isofluorane) *nu/nu* mice. Mice were monitored twice weekly for tumor growth. Animals were killed when tumors reached a mean cross-sectional area of 225 mm^2 or 400 mm^2.

In Vitro Migration and Invasion Assays. Migration chambers were preincubated in serum free media for 1 h before plating of cells. Cells (1×10^4) were plated in 24-well, 8.0-μm uncoated (BioCoat Control Inserts) and Matrigel coated (BioCoat Matrigel Coated Invasion Chambers) migration chambers (BD Biosciences). Cells were harvested 18 h post-plating. Non-translocated cells were dislodged with cotton swabs prewet with serum-free media. Translocated cells were stained with Diff-Quik per manufacturer's protocol (Dade-Behring). Soft agar growth assays were performed in 6-well dishes according to standard methods using 4×10^6 cells/well. Colonies were counted after two weeks.

Retroviral Production and Infection. Retroviral vectors expressing wild-type and *Hunk* and *Hunk K91M* were cloned and retroviral supernatants generated and used to infect mammary epithelial cell lines as described (see *SI Text*).

Immunoblotting of Hunk Protein. Protein lysates were prepared by homogenizing cell lines in EBC buffer (50 mM Tris-Hcl, pH 7.9; 120 mM NaCl; 0.5% IGEPAL; 5 mM DTT) supplemented with one tablet of Complete protease inhibitors (Roche) per 20 mL buffer, 1 μM glycerol ß-phosphate, and 0.1M NaF. Membranes were probed with anti-Hunk antibodies, and peroxidase conjugated secondary antibodies (Jackson Laboratories). Bound antibodies were detected with an enhanced chemiluminescent system (ECL+, Amersham).

Hunk in Vitro Kinase Assay. Cells were lysed at 4 °C for 40 min in lysis buffer (20 mM Tris-Hcl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 140 mM NaCl, 2 mM vanadate, 270 mM sucrose, and one tablet Complete protease inhibitor per 30 mL). Lysates were subjected to immunoprecipitation with Hunk-specific C-terminal antisera for 30 min at 4 °C. Protein A coated agarose beads were added and incubated at 4 °C for 1 h. Beads were washed twice with lysis buffer and twice with kinase buffer (50 mM Hepes pH7.4, 5 mM magnesium acetate, and 0.1 mM EGTA). Kinase assays were carried out at 30 °C for 20 min by addition of 25 mL kinase buffer with 0.1% ß-mercaptoethanol, 100 mM ATP, and 5 mCi [^{32}]ATP and were terminated by boiling for 2 min in SDS-loading buffer followed by 10% SDS/PAGE and autoradiography.

Microarray Analyses. Affymetrix microarray expression profiling and analysis was performed as described (see *SI Text*). A list of 110 genes distinguishing Hunk-wild type from Hunk-deficient c-myc-induced mouse mammary tumors was generated by comparison of Hunk-wild type and Hunk-deficient tumor samples (see Table S1). This gene set was used to classify samples from human primary breast cancer data sets into groups whose gene expression patterns resembled Hunk-wild type or Hunk-deficient myc tumors (14, 15, 18, 19). Differences in patient outcome between the two groups was assessed by the log-rank test and Cox proportional-hazards model.

ACKNOWLEDGMENTS. This work was supported by National Institutes of Health (NIH) Grant CA105490, Department of Defense (DOD) Grant W81XWH-05-1-0405, and other NIH and DOD research grants.

1. Parkin DM, Bray F, Ferlay J, Pisani P (2005) Global cancer statistics, 2002. *CA Cancer J Clin* 55:74–108.
2. Jemal A, et al. (2004) Cancer statistics, 2004. *CA Cancer J Clin* 54:8–29.
3. Fidler IJ (2003) The pathogenesis of cancer metastasis: The 'seed and soil' hypothesis revisited. *Nat Rev Cancer* 3:453–458.
4. Bernards R, Weinberg RA (2002) A progression puzzle. *Nature* 418:823.
5. Gardner HP, et al. (2000) Cloning and characterization of Hunk, a novel mammalian SNF1-related protein kinase. *Genomics* 63:46–59.
6. Gardner HP, et al. (2000) Developmental role of the SNF1-related kinase Hunk in pregnancy-induced changes in the mammary gland. *Development* 127:4493–4509.
7. Chodosh LA, et al. (2000) Protein kinase expression during murine mammary development. *Dev Biol* 219:259–276.
8. Korobko IV, Kabishev AA, Kiselev SL (1997) [Identification of the new protein kinase specifically transcribed in mouse tumors with high metastatic potential]. *Dokl Akad Nauk* 354:554–556.
9. Stewart TA, Pattengale PK, Leder P (1984) Spontaneous mammary adenocarcinomas in transgenic mice that carry and express MTV/myc fusion genes. *Cell* 38:627–637.
10. Naidu R, Wahab NA, Yadav M, Kutty MK (2002) Protein expression and molecular analysis of c-myc gene in primary breast carcinomas using immunohistochemistry and differential polymerase chain reaction. *Int J Mol Med* 9:189–196.
11. Guerin M, Barrois M, Terrier MJ, Spielmann M, Riou G (1988) Overexpression of either c-myc or c-erbB-2/neu proto-oncogenes in human breast carcinomas: Correlation with poor prognosis. *Oncogene Res* 3:21–31.
12. Deming SL, Nass SJ, Dickson RB, Trock BJ (2000) C-myc amplification in breast cancer: A meta-analysis of its occurrence and prognostic relevance. *Br J Cancer* 83:1688–1695.
13. Ma XJ, et al. (2004) A two-gene expression ratio predicts clinical outcome in breast cancer patients treated with tamoxifen. *Cancer Cell* 5:607–616.
14. Oh DS, et al. (2006) Estrogen-regulated genes predict survival in hormone receptor-positive breast cancers. *J Clin Oncol* 24:1656–1664.
15. van 't Veer LJ, et al. (2002) Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 415:530–536.
16. van de Vijver MJ, et al. (2002) A gene-expression signature as a predictor of survival in breast cancer. *N Engl J Med* 347:1999–2009.
17. Weigelt B, et al. (2003) Gene expression profiles of primary breast tumors maintained in distant metastases. *Proc Natl Acad Sci USA* 100:15901–15905.
18. Chang HY, et al. (2005) Robustness, scalability, and integration of a wound-response gene expression signature in predicting breast cancer survival. *Proc Natl Acad Sci USA* 102:3738–3743.
19. Chin K, et al. (2006) Genomic and transcriptional aberrations linked to breast cancer pathophysiologies. *Cancer Cell* 10:529–541.
20. Druker BJ, et al. (2001) Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med* 344:1031–1037.
21. Paez JG, et al. (2004) EGFR mutations in lung cancer: Correlation with clinical response to gefitinib therapy. *Science* 304:1497–1500.
22. Hardie DG, Carling D, Carlson M (1998) The AMP-activated/SNF1 protein kinase subfamily: Metabolic sensors of the eukaryotic cell? *Annu Rev Biochem* 67:821–855.
23. Suzuki A, et al. (2004) IGF-1 phosphorylates AMPK-alpha subunit in ATM-dependent and LKB1-independent manner. *Biochem Biophys Res Commun* 324:986–992.
24. Lu R, Niida H, Nakanishi M (2004) Human SAD1 kinase is involved in UV-induced DNA damage checkpoint function. *J Biol Chem* 279:31164–31170.
25. Lefebvre DL, et al. (2001) Identification and characterization of a novel sucrose-nonfermenting protein kinase/AMP-activated protein kinase-related protein kinase, *SNARK Biochem J* 355:297–305.
26. Lefebvre DL, Rosen CF (2005) Regulation of SNARK activity in response to cellular stresses. *Biochim Biophys Acta* 1724:71–85.
27. Peng CY, et al. (1998) C-TAK1 protein kinase phosphorylates human Cdc25C on serine 216 and promotes 14–3-3 protein binding. *Cell Growth Differ* 9:197–208.
28. Suzuki A, et al. (2003) ARK5 suppresses the cell death induced by nutrient starvation and death receptors via inhibition of caspase 8 activation, but not by chemotherapeutic agents or UV irradiation. *Oncogene* 22:6177–6182.
29. Suzuki A, et al. (2004) ARK5 is a tumor invasion-associated factor downstream of Akt signaling. *Mol Cell Biol* 24:3526–3535.
30. Kusakai G, et al. (2004) ARK5 expression in colorectal cancer and its implications for tumor progression. *Am J Pathol* 164:987–995.
31. Kusakai G, Suzuki A, Ogura T, Kaminishi M, Esumi H (2004) Strong association of ARK5 with tumor invasion and metastasis. J Exp Clin *Cancer Res* 23:263–268.
32. Suzuki A, et al. (2003) Induction of cell-cell detachment during glucose starvation through F-actin conversion by SNARK, the fourth member of the AMP-activated protein kinase catalytic subunit family. *Biochem Biophys Res Commun* 311:156–161.
33. Cullen PJ, Sprague GF, Jr (2000) Glucose depletion causes haploid invasive growth in yeast. *Proc Natl Acad Sci USA* 97:13619–13624.
34. Palecek SP, Parikh AS, Huh JH, Kron SJ (2002) Depression of *Saccharomyces cerevisiae* invasive growth on non-glucose carbon sources requires the Snf1 kinase. *Mol Microbiol* 45:453–469.
35. Marquis ST, et al. (1995) The developmental pattern of Brca1 expression implies a role in differentiation of the breast and other tissues. *Nat Genet* 11:17–26.
36. Leder A, Pattengale PK, Kuo A, Stewart TA, Leder P (1986) Consequences of widespread deregulation of the c-myc gene in transgenic mice: Multiple neoplasms and normal development. *Cell* 45:485–495.