Published in final edited form as:

Cancer Res. 2009 September 15; 69(18): 7198–7206. doi:10.1158/0008-5472.CAN-09-0795.

NEDD9 promotes oncogenic signaling in mammary tumor development

Eugene Izumchenko^{1,2}, Mahendra K. Singh¹, Olga V. Plotnikova^{1,3}, Nadezhda Tikhmyanova^{1,4}, Joy L. Little¹, Ilya G. Serebriiskii¹, Sachiko Seo⁵, Mineo Kurokawa⁵, Brian L. Egleston¹, Andres Klein-Szanto¹, Elena N. Pugacheva⁶, Richard R. Hardy⁷, Marina Wolfson², Denise C. Connolly⁸, and Erica A. Golemis^{1,*}

¹Program in Molecular and Translational Medicine, Fox Chase Cancer Center, Philadelphia, PA 19111

²Faculty of Health Sciences, Ben Gurion University of the Negev, Beer Sheva, Israel

³Department of Molecular Biology and Medical Biotechnology, Russian State Medical University, Moscow, Russia

⁴Department of Biochemistry, Drexel University School of Medicine, Philadelphia, PA 19102

⁵Department of Hematology and Oncology, Graduate School of Medicine, University of Tokyo, Tokyo, Japan

⁶Mary Babb Randolph Cancer Center, West Virginia University, Morgantown, WV 26506

⁷Program in Immunology, Fox Chase Cancer Center, Philadelphia, PA, 19111

⁸Program in Women's Cancer, Fox Chase Cancer Center, Philadelphia, PA, 19111

Abstract

In the past 3 years, altered expression of the HGF1/CAS-L/NEDD9 scaffolding protein has emerged as contributing to cancer metastasis in multiple cancer types. However, while some studies have identified elevated NEDD9 expression as pro-metastatic, other work has suggested a negative role in tumor progression. We here show that the *Nedd9* null genetic background significantly limits mammary tumor initiation in the MMTV-polyoma virus middle T (PyVmT) genetic model. Action of *Nedd9* is tumor cell intrinsic, with immune cell infiltration, stroma, and angiogenesis unaffected. The majority of the late-appearing mammary tumors of MMTV-PyVmT;*Nedd9*^{-/-} mice are characterized by depressed activation of proteins including AKT, SRC, FAK, and ERK, emphasizing an important role of *Nedd9* as a scaffolding protein for these pro-oncogenic proteins. Analysis of cells derived from primary *Nedd9*^{+/+} and *Nedd9*^{-/-} tumors demonstrated persistently reduced FAK activation, attachment, and migration, consistent with a role for NEDD9 activation of FAK in promoting tumor aggressiveness. This study provides the first *in vivo* evidence of a role for NEDD9 in breast cancer progression, and suggests that *Nedd9* expression may provide a biomarker for tumor aggressiveness.

Introduction

Human breast cancer is a heterogeneous disease characterized by variable response to treatment and differences in prognosis (1), with mortality commonly linked to the existence of distant

*corresponding author: Erica A. Golemis Fox Chase Cancer Center, W406 333 Cottman Ave. Philadelphia, PA 19111 USA (215) 728-2860 ph, -3616 fax EA_Golemis@fccc.edu.

metastases upon diagnosis (2). It is well established that primary lesions inducing the aberrant expression or activity of proteins such as the transmembrane receptor ErbB2/HER2, the estrogen receptor (ER), and the classic tumor suppressors such as p53 contribute to breast cancer incidence and progression. More recently, altered expression or activation of additional proteins such as FAK (3), AKT (4), SHCA (5), integrins (6), and other proteins operating in signaling networks associated with cell survival and metastasis have also been implicated in modulating breast cancer disease course. Although in some cases these proteins are mutationally activated, in other cases activity is enhanced without clearly responsible genetic lesions. In these cases, it is likely that activity of core cancer-promoting signaling proteins is induced by changes in as yet poorly defined signaling partners, which augment their function to promote transformation.

The HEF1(7)/CAS-L(8)/NEDD9 non-catalytic scaffolding protein is best known for its roles in coordinating the FAK and SRC signaling cascades relevant to integrin dependent adhesion, migration, and survival (9-12). Recently, functional interactions between NEDD9 and Rac have been shown to be necessary for mesenchymal movement in melanoma cell motility and invasion (13). DNA amplification or transcriptional up-regulation of the *NEDD9* gene, leading to elevated expression of the NEDD9 protein, has been reported as a potent regulator of cancer progression, invasion, and metastasis in melanoma (14) and glioblastoma (15), and linked to metastasis of LKB1^{-/-} lung tumors (16). As such, NEDD9 expression changes have been proposed as biomarkers for tumor aggressiveness.

NEDD9 is abundantly expressed in many breast cancer cell lines (17) and hyper-phosphorylation associated with activation of NEDD9 has been detected in phosphoproteome analysis of heregulin-stimulated ErbB2-positive breast adenocarcinoma MCF7 cells (18). However, an *in vivo* role for *Nedd9* in breast cancer has not been established. Although *Nedd9* overexpression clearly promotes migration and invasion in MCF7 cells (12) and other cancer cell lines (14,15), siRNA depletion of *Nedd9* identified this gene as an inhibitor of migration in untransformed MCF-10A breast epithelial cells (19). Further, an independent study identified down- rather than up-regulation of *Nedd9* as part of a transcriptional signature associated with enhanced metastasis to the lung in a TGF- β -associated mammary cancer model (20). These conflicting results raise the possibility that at least in some cell types, it is loss of *Nedd9* rather than overexpression of *Nedd9* is tumor promoting, comparable to the complex cell type-specific activity of proteins such as APC, which can act either as an oncogene or tumor suppressor (21).

The recent development of a viable, fertile *Nedd9* knockout strain (22) provided the opportunity to directly evaluate the role of *Nedd9* in mammary cancer initiation and progression. The polyoma virus middle T antigen (PyVmT) antigen induces tumorigenesis based in large part on its binding and activation of the proteins SHC, SRC, and PI3K, which are central effectors of ErbB2/HER2 (reviewed in (23)). Detailed pathological analysis of PyVmT tumors indicates progression from pre-malignant to highly malignant stages is very similar to that seen in human breast tumors (24), and a large-scale microarray profiling study has confirmed that cancers arising from overexpression of PyVmT, HER2/ErbB2, and Ras showed tightly clustered gene expression profiles that were distinct from those associated with Myc- or SV40 T antigen-initiated tumors, further confirming relevance to human disease (25). In this study, we have used the MMTV-PyVmT oncogenic model to compare mammary tumor progression in *Nedd9* wild type versus null genetic backgrounds. Our data indicate that lack of *Nedd9* significantly limits tumor incidence and oncogenic signaling in mammary tumors. Surprisingly, mammary tumor growth in *Nedd9* null mice show differences from *Nedd9* wild type mice from the time pre-malignant lesions are first detectable, in contrast to previous suggestions of a role for this protein in metastasis; and also show that these differences are

linked to reduced activation of multiple signaling pathways linked to tumor cell growth and invasion in *Nedd9* null mice.

Materials and Methods

Mouse strains, handling, measurement of tumors

All experiments involving mice were pre-approved by the FCCC Institutional Animal Care and Use Committee (IACUC). MMTV-PyVmT mice (26) of the 634Mul/J subline were purchased from Jackson Laboratories (Bar Harbor, ME). Homozygous C57BL/6 *Nedd9*^{-/-} females (22) were crossed with MMTV-PyVmT males, then female *Nedd9*^{+/-} offspring were mated with *Nedd9*^{+/-};PyVmT-positive males to generate virgin female siblings for analysis.

Tumor growth assessment

Mice were palpated twice weekly to assess mammary tumor onset. Tumor volume was calculated following caliper measurement as width × length × 0.4. Mice were euthanized by methoxy-fluorane (Metofane) inhalation when the longest dimension of the largest tumor reached 2 cm or if mice exhibited signs of illness or distress. The largest tumor and lungs were excised, divided, and processed for Western analysis and pathology. Pathology specimens were fixed overnight in paraformaldehyde, paraffin-embedded, sectioned, and analyzed by hematoxylin and eosin (H & E) staining (Sigma-Aldrich, St. Louis, MO). Tumor sections were immunostained with antibodies to Mac2 (Cedarlane, Tornmy, Canada), B220-CD45R (PharMingen, San Diego, CA), CD31, CD3, and Ki-67 (DAKO, Carpinteria, CA). Images were acquired using a Nikon Eclipse E600 microscope. Lung metastases were expressed as number of metastases/mm² of lung cross-section, using Image Pro-Plus (Media Cybernetics, Silver Springs, MD). All analyses were performed by a board-certified pathologist (AK-S) blind to sample identity.

For Figure 1a, we used Kaplan Meier curves with log-rank tests. For figure 1b, we used semi-parametric regression (27). For Figure 1c, we used Poisson regression estimated by generalized estimating equations (GEE) assuming a Markov correlation structure (28) and including age in the model via restricted cubic splines (29) and interacting it with *Nedd9* group. For Supp. Figure 1 and Figures 5C, and 5D, we used similar GEE-estimated linear regressions with appropriate correlation structures. For Suppl. Table 1, and Figures 1D, 3B, 3C, 5B, and 6B, we used Wilcoxon rank-sum tests. For Figure 4d, we fit simple linear regressions of age with ordinal biomarker covariates (low, medium, high, wild type). Robust standard errors were used for regressions. Error bars in figures represent +/- 1 standard error.

Early lesions

MMTV-PyVmT;*Nedd9*^{-/-} and MMTV-PyVmT;*Nedd9*^{+/+} animals were euthanized at 4 and 6 weeks of age, mammary glands were paraffin-embedded and stained for H&E, Ki-67, phosphorylated FAK-Y³⁹⁷ and Erk1/2-T²⁰²/Y²⁰⁴. For whole mount analysis, excised thoracic mammary glands attached to glass slides were treated with acetone overnight, then immersed in 70% ethanol for 1h, washed with dH₂O, and put in alum carmine solution (Fisher Scientific, Pittsburgh, PA) overnight. Sections were de-stained in increasing ratios of ethanol to dH₂O for 30 minutes, treated with Histoclear overnight, and photographed.

Western analysis of tumor lysates

Tumor sections histologically confirmed to contain >90% tumor tissue were harvested, homogenized, and lysed in PBS-TDS buffer (1×PBS, 1% Triton X-100, 0.1% SDS, 20% glycerol) containing complete protease and phosphatase inhibitor cocktail (Roche Diagnostic, Mannheim, Germany). Whole cell lysates from the human breast cancer cell line MCF7 were

used as positive loading controls. Primary antibodies used recognized *Nedd9* (2G9) (30), p130Cas (Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated FAK-Y³⁹⁷, ShcA-Y³¹⁷, and Src-Y⁴¹⁸, FAK, Src and ShcA (Abcam, Cambridge, MA), phosphorylated Erk1/2-T²⁰²/Y²⁰⁴, AKT-S⁴⁷⁵ and AKT-T³⁰⁸, and Erk1/2 and AKT (Cell Signaling, Danvers, MA), and β -actin (Sigma, St. Louis, MO). Secondary horseradish peroxidase (HRP)-conjugated antibodies were from Amersham Biosciences (Piscataway, NJ). Proteins were visualized using the West-Pico system (Pierce, Rockford, IL). Image analysis was done using NIH Image-J (National Institutes of Health, Bethesda, MD), with signal intensity normalized to β -actin.

Derivation of cells

Tumors were dissected, rinsed with PBS and minced, then treated with 0.2% collagenase for 2 hours at 37°C. Cells were washed several times with serum free DMEM, and finally with low calcium medium supplemented with 5% horse serum. Tissues kept in low calcium medium were transferred to T-25 flasks coated with 0.1% gelatin and incubated in a 37°C incubator overnight. The next day, supernatants containing floating cells were transferred and seeded into new flasks, and maintained with regular media changes until confluency, to prevent fibroblast growth. Fibroblast-free cell populations were derived typically after 6-8 weeks. *In vitro* proliferation was measured by seeding approximately 1×10^5 cells on 0.1% gelatin-coated T25 flasks. At specific time points, cells were trypsinized and counted using Trypan blue exclusion analysis. All analyses used cells passaged <6 times.

Cell spreading, migration, proliferation, and anoikis

Cells were fixed in 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, and blocked with 3% BSA in PBS before incubation with antibodies. Epifluorescence was measured using an inverted Nikon TE300 microscope (Melville, NY), with 12-bit images acquired using a Spot RT monochrome camera (Diagnostic Instruments, Sterling Heights, MI). To measure cell attachment, α -paxillin mAb was used to outline cells, and cell area measurements made using MetaMorph or MetaVue software (Molecular Devices, Universal Imaging, Downingtown, PA) software to score pixels within cell perimeters. Cell motility was monitored with a Nikon TE300 microscope using 10x NA 0.25 Plan A objective, and images were collected with a CCD video camera (Roper Scientific, Trenton, NJ) at 20-min intervals over a 12-h period and then digitized and stored as image stacks using MetaMorph. For wound-healing assays, cell monolayers were wounded with pipette tips, then cells incubated at 37°C on the stage of a Nikon Eclipse E800 inverted microscope (Carl Zeiss, Thornwood, NY, USA), while images were acquired for 12 h with a Quantix-cooled CCD camera (Roper Scientific, Trenton, NJ, USA). For anoikis assays, cells were grown on poly-HEMA (Sigma-Aldrich, St. Louis, MO), then apoptosis measured by annexin-V staining using a kit from Guava Technologies (Hayward, CA). Lysates were prepared using standard procedures. Caspase-3 antibodies were obtained from Cell Signaling (Danvers, MA).

Results

Nedd9^{-/-} status limits early tumor development in MMTV-PyVmT-induced mammary tumors

We compared the incidence and latency of mammary tumor appearance in *Nedd9*^{-/-} or *Nedd9*^{+/+} mice in the presence or absence of the MMTV-PyVmT transgene (Figure 1). No animals lacking the MMTV-PyVmT transgene developed mammary tumors, in a study group maintained until 1 year of age. However, *Nedd9*^{-/-} status significantly limited multiple parameters of MMTV-PyVmT-induced early mammary tumor development and progression, including average age at initial detection of tumors (62 days for *Nedd9*^{-/-} versus 51 days post-natal for *Nedd9*^{+/+}) (Fig 1A). There was also a reduction in total tumor burden (Fig 1B), number of independent tumors (Fig 1C) and growth rate of the largest single tumor (Supp Fig 1). *Nedd9*^{-/-} status also was associated with a trend towards fewer lung metastases (Fig 1D).

However, this was not statistically significant, given the very low overall numbers of metastases detectable at the time the volume of the primary mammary tumors necessitated euthanasia.

***Nedd9*^{-/-} status does not affect normal mammary gland development, but delays tumor formation**

The limiting effect of loss of *Nedd9* on initial mammary tumor development might reflect intrinsic differences in mammary gland development that limit tumor precursor cell populations, or differences in tumor development in a comparable mammary gland microenvironment. Comparison of whole mount and paraffin-embedded specimens of mammary glands from adolescent, young adult, pregnant, lactating, and post-involution mice indicated no detectable differences between *Nedd9*^{-/-} and *Nedd9*^{+/+} animals (Supp Fig 2 and not shown). However, MMTV-PyVmT-induced pre-tumorous lesions appeared earlier in *Nedd9*^{+/+} mice (Fig 2A), and fewer independent primary lesions were observed in *Nedd9*^{-/-} animals at both 4- and 6-week time points. Ki67 staining indicated a lower percentage of proliferating cells in the mammary tumors in MMTV-PyVmT;*Nedd9*^{-/-} versus MMTV-PyVmT;*Nedd9*^{+/+} mice at both 4 and 6 weeks of age (Figs 2A and 2B). Histopathological analysis indicated no qualitative morphological differences between the comparably staged early mammary tumors of MMTV-PyVmT;*Nedd9*^{-/-} and MMTV-PyVmT;*Nedd9*^{+/+} mice, bolstering the idea that the primary difference at the early time point predominantly reflected differences in growth rate. Finally, analysis of Ki-67 staining in tumors at the time of euthanasia indicated overall differences in proliferation seen in early lesions were no longer apparent (Fig 2C).

The reduced tumor growth in *Nedd9*^{-/-} mice reflects tumor-intrinsic action

NEDD9 has been implicated in endothelial cell signaling (31) and *Nedd9*^{-/-} mice have specific defects in immune system maturation and migration (22), raising the possibility that consequences of loss of *Nedd9* on tumor growth might in part reflect effects of the microenvironment. However, immunohistochemical (IHC) staining for CD31 to visualize vascularization of mammary tumors in MMTV-PyVmT;*Nedd9*^{+/+} versus MMTV-PyVmT;*Nedd9*^{-/-} mice indicated no significant differences (Figs 3A,B). IHC staining for markers of T cells (CD3), B cells (CD45R) and macrophages (Mac2) indicated no *Nedd9*-dependent difference in the infiltration of these cells into tumors (Figs 3C,D). Together, these data supported a tumor-intrinsic effect of *Nedd9*^{-/-} genotype.

Reduced activity of multiple pro-oncogenic signaling pathways in MMTV-PyVmT;*Nedd9*^{-/-} tumors correlates with time of tumor appearance

NEDD9 directly binds and provides scaffolding activity for activation of the pro-invasive integrin effectors FAK and Src (Fig 4A) (10). Strikingly, activation-associated phosphorylation of these proteins (phospho-FAK-Y³⁹⁷, and phospho-Src-Y⁴¹⁸) was reduced or undetectable in the majority of MMTV-PyVmT;*Nedd9*^{-/-} tumors (17/21 tumors for FAK, and 15/21 for Src) relative to tumors from MMTV-PyVmT;*Nedd9*^{+/+} animals (5/15 tumors for FAK, and 4/15 for Src)(Figs 4B, C). The set of tumors with reduced FAK activation overlapped almost entirely with the set of tumors with reduced Src activation, reflecting the interdependence of the two activation processes. Total expression levels of FAK and Src were not affected by *Nedd9* status.

NEDD9 has partially overlapping function with its paralog p130Cas/BCAR1 (32), which is also expressed in mammary cells, and can both dimerize with NEDD9 and sustain activation of FAK and Src (7,33). While variance in p130Cas levels as a consequence of *Nedd9* deletion might explain the heterogenous activation of FAK and Src in PyVmT;*Nedd9*^{-/-} tumors (Fig 4B), p130Cas levels were consistent in all tumor lysates, regardless of *Nedd9* genotype.

In addition to their roles in cell migration and invasion, FAK and Src phosphorylate and activate the ShcA adaptor, an upstream activator of the Ras pathway (34). Hence, *Nedd9* could potentially be required for activation of Shc-dependent signaling proteins that influence proliferation (Fig 4A), relevant to the reduced proliferation phenotype of the early *Nedd9*^{-/-} lesions (Fig 2). Indeed, activation of Erk1/2 (phospho-Erk-T^{202/-}Y²⁰⁴), ShcA (phospho-ShcA-Y³¹⁷) and AKT (phospho-AKT-T³⁰⁸, phosphorylated by the PI3K effector PDK1 (37), and -S⁴⁷³ auto-phosphorylation (35), dependent in part on FAK) was reduced or absent in many *Nedd9*^{-/-} versus *Nedd9*^{+/+} tumors (Fig 4B). The subset of *Nedd9*^{-/-} tumors with reduced FAK or Src activation overlapped very significantly with the set of tumors with reduced SHC phosphorylation, and also correlated well with the set of tumors with reduced AKT phosphorylation. Given these results and the known close connection between FAK and SHC, we investigated whether NEDD9 associated with SHC, and indeed, identified robust co-immunoprecipitation of these proteins in vivo, supporting a direct link relevant to control of cell proliferation (Supp Fig 3). Some overlap was also seen between the tumor sets lacking FAK, SRC, and ERK1/2 activation, although not to the same degree as with SHC or AKT; this likely reflects the many diverse cellular signaling pathways contributing to ERK activation. Finally, for both phospho-FAK-Y³⁹⁷ and phospho-Erk-T^{202/-}Y²⁰⁴, we confirmed that the *Nedd9*-dependent differences we observed in the tumor cell lysates were also detectable by immunohistochemistry specifically in tumor cells (Supp Fig 4), in congruence with other data (Fig 3) indicating that the action of *Nedd9* was tumor-intrinsic.

Importantly, segregation of individual MMTV-PyVmT;*Nedd9*^{-/-} mice into groups characterized by low, intermediate, or high levels of activation of these proteins revealed significant trends in which low pathway activation correlated with later tumor onset (Fig 4D). While the average latency until tumor appearance was 62 days for *Nedd9*^{-/-} versus 51 days for *Nedd9*^{+/+} animals, latency was delayed until 66 days or more for the group of tumors with the lowest signaling pathway activation. These results strongly supported the idea that activation of these *Nedd9*-dependent pathways was relevant to the requirement of *Nedd9* for efficient tumor development.

***Nedd9*^{-/-} cell lines have reduced FAK activation, spreading, migration, but complex cell survival properties**

Because of the central relation of FAK-NEDD9 interactions to NEDD9 impact on multiple pro-oncogenic signaling cascades, we next assessed 3 independent MMTV-PyVmT;*Nedd9*^{-/-} versus 3 MMTV-PyVmT;*Nedd9*^{+/+}-derived tumor cell lines to determine whether failure to activate FAK was a persistent and functionally important in this physiological context. Although both *Nedd9*^{+/+} and *Nedd9*^{-/-} cells formed paxillin-positive focal adhesions (Figure 5A), these were morphologically distinct between the two cell types, with larger and more clustered focal adhesions (associated with reduced motility) seen in *Nedd9*^{-/-} cells. Strikingly, few of the focal adhesions in *Nedd9*^{-/-} cells were positive for activated phospho-FAK-Y³⁹⁷, while most of those observed in *Nedd9*^{+/+} cell lines were phospho-FAK-Y³⁹⁷ positive, confirming FAK activation remained dependent on Nedd9 in cancer-derived cell lines.

In overexpression and siRNA depletion models, Nedd9/FAK/Src interactions have been shown to regulate cell attachment, migration, and detachment-associated cell survival (anoikis) (9, 10). Initial attachment of cells to fibronectin was very significantly reduced in *Nedd9*^{-/-} cell lines (Figure 5B). Further, two discrete cell migration assays revealed very significantly decreased rates of cell migration in *Nedd9*^{-/-} cells (Figures 5C,D), with many *Nedd9*^{-/-} cells showing little or no motility. Further, growth of cell lines on poly-HEMA to prevent attachment was associated with earlier and enhanced appearance of the apoptotic markers annexin-V (Figure 6A) and cleaved caspase-3 (Figure 6B) in MMTV-PyVmT;*Nedd9*^{-/-} cell lines,

suggesting greater susceptibility to anoikis, compatible with their consistently reduced activation of FAK.

***Nedd9* null mice do not spontaneously develop solid tumors, but have late-appearing hematological defects**

Although *Nedd9*^{-/-} mice do not have pronounced developmental defects (22), the abnormally low activation of essential cellular signaling pathways seen in the majority of MMTV-PyVmT;*Nedd9*^{-/-} tumors suggested that the sustained lack of *Nedd9* might ultimately independently select for alternative survival pathways promoting malignancy. However, among cohorts of *Nedd9*^{+/+}, *Nedd9*^{+/-}, and *Nedd9*^{-/-} littermates aged to 1 year, no animals showed signs of illness, and no spontaneous solid tumors were detected after complete autopsies and histopathological studies of multiple tissues. Mammary tissue from year-old animals was indistinguishable between animals with different *Nedd9* genotypes (not shown). Extensive literature suggests an important role for *Nedd9* in signaling related to normal differentiation and function of cells in the hematopoietic system (8,22,36,37). Indeed, histopathological analysis indicated that 9/11 *Nedd9*^{-/-} mice and 8/11 *Nedd9*^{+/-} mice exhibited reactive lymphoid hyperplasia affecting solid tissues, in contrast to only 2/11 *Nedd9*^{+/+} mice. 2/11 *Nedd9*^{-/-} mice had signs of early lymphomas involving B or T cells (Supp Fig 5A). Analysis of peripheral blood cells (Supp Table 1) and analysis of blood cells within tissues indicated subtle but statistically significant distortion in some cell populations in *Nedd9*^{-/-} versus *Nedd9*^{+/+} mice (Supp Table 1, Supp Fig 5B, C). In particular, decreased B cells, but increased macrophages were found in the peripheral blood and spleen of *Nedd9*^{-/-} in comparison to *Nedd9*^{+/+} mice (Supp Table 1). This suggests long time imbalance of *Nedd9*-dependent signaling is sufficient either to trigger pro-inflammatory and/or pre-neoplastic changes in immune system cells, or to bias clonal outgrowth of specific cell populations based on a defect in the immune system microenvironment. One possibility for the different dependence on *Nedd9* in different tissues may be varying abundance of either specific NEDD9 paralogs (e.g. (38,39)) or partner proteins, leading to different signaling outcomes.

Discussion

Our results reveal that NEDD9 is a cancer cell-intrinsic protein that contributes to mammary tumor development in the MMTV-PyVmT mouse model. In reference to other genetic modifiers that have been tested in the MMTV-PyVmT model, the effect of *Nedd9* null status is not as dramatic as that seen with deletion of its binding partner FAK (40), but comparable to or greater than that observed in mice null for the important breast cancer signaling proteins PAR1 (41), MEKK1 (42), CD44 (43), and others. These results suggest that *Nedd9* expression may be an important modulator of breast cancer incidence in humans.

Although our data are compatible with the pro-oncogenic role identified for *Nedd9* overexpression in glioblastoma, melanoma, and lung cancers (14-16), they differ in important ways. In particular, lack of *Nedd9* reduces proliferation from very early stages of tumor development, rather than acting at the point of invasion and metastasis, as suggested by other studies. This reduced rate of tumor onset likely reflects an important role for *Nedd9* in maintaining activation not only of its direct partners, FAK and SRC, but also the key Ras effectors AKT, SHCA, and ERK1/2. The striking correlation of the degree to which these pathways are activated with the time of tumor detection argues that supporting this activation is a critical early action of NEDD9. In this context, the novel interaction between NEDD9 and SHC we report here provides a direct means by which NEDD9 supports Ras/Raf pathway activation. Together with the fact that an earlier study has demonstrated that NEDD9-dependent tumor promotion has previously been shown to be partly dependent on Ras/Raf

pathway activation (14), these data suggest close coordination rather than strict unidirectional epistasis explains the relation of NEDD9 and Ras signaling in tumor growth.

Given our results, the opposite suggestion by Minn et al, that down-regulation of *Nedd9* is part of a signature for breast cancer metastasis (20), may reflect the fact that *Nedd9* is required at early stages in the tumor process, but downregulated after metastasis. Indeed, MMTV-PyVmT;*Nedd9*^{-/-} mammary tumor cells lines are less migratory than MMTV-PyVmT;*Nedd9*^{+/+} cells, in agreement with our earlier observations that NEDD9 positively regulates cell migration and matrix metalloproteinase expression in transformed breast adenocarcinoma cells (12), but in opposition to the report that NEDD9 negatively regulates migration in normal mammary epithelial cells (19). Alternatively, the different findings may reflect a distinct feature of the TGF- β model employed in the Minn study. NEDD9 directly binds and influences the function of TGF- β effectors including SMAD3 (44) and other SMADs (45), making it plausible that NEDD9 might act differently in a TGF- β —driven tumor. Epithelial-mesenchymal transition (EMT) is an important consequence of increased TGF- β signaling in tumor metastasis. In support of the present study, we have examined a number of hallmarks of TGF- β -induced EMT in *Nedd9*^{-/-} tumors and tumor-derived cell lines, including upregulation of vimentin and downregulation of E-cadherin. No significant differences were detected in the MMTV-PyVmT model (unpublished results). Finally, the difference between our data and those reported by Minn may reflect a difference observed at the mRNA level that does not affect NEDD9 protein levels, as we and others have shown that NEDD9 undergoes significant post-translational regulation during cell division and cell death (9,46). More study of these issues is required.

Interestingly, our data indicate that continued expression of the NEDD9 paralog BCAR1/p130Cas is unable to compensate for *Nedd9* null status, even though transgenic overexpression of p130Cas activates SRC and AKT and promotes mammary tumor progression in a mouse model (47). A possible explanation lies in the typically non-dynamic expression level of p130Cas under different growth conditions. In contrast, NEDD9 is strikingly up-regulated in highly proliferating cells (17) and may be required to support the proliferation of such cells. Levels of p130Cas were constant in tumors derived from *Nedd9*^{-/-} and *Nedd9*^{+/+} mice (Figure 4), suggesting that even under strong selective conditions, upregulation of this protein was either non-tumor promoting or impossible in a *Nedd9*^{-/-} background. Another possibility is that *Nedd9* induces additional signaling pathways that are not influenced by p130Cas, which contribute specifically to tumor growth. In interesting contrast, *p130Cas*^{-/-} status is embryonally lethal (day 11.5) (48), while *Nedd9*^{-/-} status is not associated with known pre-natal defects, emphasizing the different requirements for signaling proteins in tumors versus normal development.

Large-scale studies of the breast cancer and other cancer genomes have begun to unearth a large number of potential pro-oncogenic mutations targeting genes previously not known to be important for cancer. These genes are not identical in different cancer types, or in a given type of cancer arising in different individuals. In parallel, systems biology studies of large biological networks (49) have suggested that activity of cancer signaling pathways can be enhanced by inappropriate activation of proteins acting at various points within a pathway, and that such activation can arise not only from mutations, but also from epigenetic, post-transcriptional, or post-translational events that alter the signaling landscape of a cell. Together, these findings suggest that proteins that broadly influence pathway activation status may have important actions in conditioning cancer initiation and progression. Since NEDD9 is not only coupled to the cell adhesion and migration machinery (9,32), but also contributes to normal mitotic progression (50) and mediates proliferative and survival signaling (46), we believe it is likely that the dynamic upregulation of NEDD9 may represent convenient means for cancer signaling networks to coordinately activate multiple pathways useful for tumor growth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Susan Shinton, Sharon Howard, Cynthia Spittle, Emmanuelle Nicolas, Anthony Lerro, Jackie Valvardi, Fangping Chen, and Catherine Renner (from Fox Chase Cancer Center) and Nicolas Day (from Germantown Academy) for technical help. This work was supported by NIH RO1s CA63366 and CA113342; W81XWH-07-1-0676 from the Army Materiel Command; and Pennsylvania Tobacco Settlement funding (to EAG); by the Israel Cancer Association and Stanley Abersur Research Foundation (to MW); and by NIH core grant CA-06927 and the Pew Charitable Fund (to Fox Chase Cancer Center). EI was supported by the American Associates, Ben-Gurion University of the Negev; JL by NIH T32 CA-009035; DCC by NCI SPORE PA50 CA-083638.

References

1. Perou CM, Sorlie T, Eisen MB, et al. Molecular portraits of human breast tumours. *Nature* 2000;406(6797):747–52. [PubMed: 10963602]
2. Rugo HS. The importance of distant metastases in hormone-sensitive breast cancer. *Breast (Edinburgh, Scotland)* 2008;17(Suppl 1):S3–8.
3. Lahlou H, Sanguin-Gendreau V, Zuo D, et al. Mammary epithelial-specific disruption of the focal adhesion kinase blocks mammary tumor progression. *Proc Natl Acad Sci U S A* 2007;104(51):20302–7. [PubMed: 18056629]
4. Maroulakou IG, Oemler W, Naber SP, Tschlis PN. Akt1 ablation inhibits, whereas Akt2 ablation accelerates, the development of mammary adenocarcinomas in mouse mammary tumor virus (MMTV)-ErbB2/neu and MMTV-polyoma middle T transgenic mice. *Cancer Res* 2007;67(1):167–77. [PubMed: 17210696]
5. Ursini-Siegel J, Hardy WR, Zuo D, et al. ShcA signalling is essential for tumour progression in mouse models of human breast cancer. *EMBO J* 2008;27(6):910–20. [PubMed: 18273058]
6. Guo W, Pylayeva Y, Pepe A, et al. Beta 4 integrin amplifies ErbB2 signaling to promote mammary tumorigenesis. *Cell* 2006;126(3):489–502. [PubMed: 16901783]
7. Law SF, Estojak J, Wang B, Mysliwiec T, Kruh GD, Golemis EA. Human Enhancer of Filamentation 1 (HEF1), a novel p130Cas-like docking protein, associates with FAK, and induces pseudohyphal growth in yeast. *Mol Cell Biol* 1996;16:3327–37. [PubMed: 8668148]
8. Minegishi M, Tachibana K, Sato T, Iwata S, Nojima Y, Morimoto C. Structure and function of Cas-L, a 105-kD Crk-associated substrate-related protein that is involved in beta-1 integrin-mediated signaling in lymphocytes. *J Exp Med* 1996;184:1365–75. [PubMed: 8879209]
9. O'Neill GM, Golemis EA. Proteolysis of the docking protein HEF1 and implications for focal adhesion dynamics. *Mol Cell Biol* 2001;21:5094–108. [PubMed: 11438665]
10. O'Neill GM, Seo S, Serebriiskii IG, Lessin SR, Golemis EA. A new central scaffold for metastasis: parsing HEF1/Cas-L/NEDD9. *Cancer Res* 2007;67(19):8975–9. [PubMed: 17908996]
11. van Seanter GA, Salman HJ, Law SF, et al. Focal adhesion kinase regulates beta1 integrin dependent migration through an HEF1 effector pathway. *Eur J Imm* 2001;31:1417–27.
12. Fashena SJ, Einarson MB, O'Neill GM, Patriotis CP, Golemis EA. Dissection of HEF1-dependent functions in motility and transcriptional regulation. *J Cell Sci* 2002;115:99–111. [PubMed: 11801728]
13. Sanz-Moreno V, Gadea G, Ahn J, et al. Rac activation and inactivation control plasticity of tumor cell movement. *Cell* 2008;135(3):510–23. [PubMed: 18984162]
14. Kim M, Gans JD, Nogueira C, et al. Comparative oncogenomics identifies NEDD9 as a melanoma metastasis gene. *Cell* 2006;125(7):1269–81. [PubMed: 16814714]
15. Natarajan M, Stewart JE, Golemis EA, et al. HEF1 is a necessary and specific downstream effector of FAK that promotes the migration of glioblastoma cells. *Oncogene* 2006;25(12):1721–32. [PubMed: 16288224]
16. Ji H, Ramsey MR, Hayes DN, et al. LKB1 modulates lung cancer differentiation and metastasis. *Nature* 2007;448(7155):807–10. [PubMed: 17676035]

17. Law SF, Zhang Y-Z, Klein-Szanto A, Golemis EA. Cell-cycle regulated processing of HEF1 to multiple protein forms differentially targeted to multiple compartments. *Mol Cell Biol* 1998;18:3540–51. [PubMed: 9584194]
18. Nagashima T, Oyama M, Kozuka-Hata H, Yumoto N, Sakaki Y, Hatakeyama M. Phosphoproteome and transcriptome analyses of ErbB ligand-stimulated MCF-7 cells. *Cancer genomics & proteomics* 2008;5(34):161–8. [PubMed: 18820370]
19. Simpson KJ, Selfors LM, Bui J, et al. Identification of genes that regulate epithelial cell migration using an siRNA screening approach. *Nat Cell Biol.* 2008
20. Minn AJ, Gupta GP, Siegel PM, et al. Genes that mediate breast cancer metastasis to lung. *Nature* 2005;436(7050):518–24. [PubMed: 16049480]
21. Nathke IS. The adenomatous polyposis coli protein: the Achilles heel of the gut epithelium. *Annu Rev Cell Dev Biol* 2004;20:337–66. [PubMed: 15473844]
22. Seo S, Asai T, Saito T, et al. Crk-associated substrate lymphocyte type is required for lymphocyte trafficking and marginal zone B cell maintenance. *J Immunol* 2005;175(6):3492–501. [PubMed: 16148091]
23. Dilworth SM. Polyoma virus middle T antigen and its role in identifying cancer-related molecules. *Nat Rev Cancer* 2002;2(12):951–6. [PubMed: 12459733]
24. Lin EY, Jones JG, Li P, et al. Progression to malignancy in the polyoma middle T oncoprotein mouse breast cancer model provides a reliable model for human diseases. *Am J Pathol* 2003;163(5):2113–26. [PubMed: 14578209]
25. Desai KV, Xiao N, Wang W, et al. Initiating oncogenic event determines gene-expression patterns of human breast cancer models. *Proc Natl Acad Sci U S A* 2002;99(10):6967–72. [PubMed: 12011455]
26. Guy CT, Cardiff RD, Muller WJ. Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. *Mol Cell Biol* 1992;12(3):954–61. [PubMed: 1312220]
27. Ruppert, D.; Wand, MP.; Carroll, RJ. Semiparametric regression. Cambridge University Press; New York: 2003.
28. Shults J, Ratcliffe SJ, Leonard M. Improved generalized estimating equation analysis via xtqls for quasi-least squares in STATA. *The STATA Journal* 2007;7:147–66.
29. Harrell, FE. Regression Modeling Strategies. Springer; New York: 2001. Chapter 2
30. Pugacheva EN, Golemis EA. The focal adhesion scaffolding protein HEF1 regulates activation of the Aurora-A and Nek2 kinases at the centrosome. *Nat Cell Biol* 2005;7(10):937–46. [PubMed: 16184168]
31. Tang H, Hao Q, Fitzgerald T, Sasaki T, Landon EJ, Inagami T. Pyk2/CAKbeta tyrosine kinase activity-mediated angiogenesis of pulmonary vascular endothelial cells. *J Biol Chem* 2002;277(7):5441–7. [PubMed: 11739395]
32. O'Neill GM, Fashena SJ, Golemis EA. Integrin signaling: a new Cas(t) of characters enters the stage. *Trends Cell Biol* 2000;10:111–9. [PubMed: 10675905]
33. Law SF, Zhang Y-Z, Fashena S, Toby G, Estojak J, Golemis EA. Dimerization of the docking/adaptor protein HEF1 via a carboxy-terminal helix-loop-helix domain. *Exp Cell Res* 1999;252:224–35. [PubMed: 10502414]
34. Blake RA, Broome MA, Liu X, et al. SU6656, a selective src family kinase inhibitor, used to probe growth factor signaling. *Mol Cell Biol* 2000;20(23):9018–27. [PubMed: 11074000]
35. Tokar A, Newton AC. Akt/protein kinase B is regulated by autophosphorylation at the hypothetical PDK-2 site. *J Biol Chem* 2000;275(12):8271–4. [PubMed: 10722653]
36. Nakamoto T, Seo S, Sakai R, et al. Expression and tyrosine phosphorylation of Crk-associated substrate lymphocyte type (Cas-L) protein in human neutrophils. *J Cell Biochem* 2008;105(1):121–8. [PubMed: 18465784]
37. Astier A, Manie S, Avraham H, et al. The related adhesion focal tyrosine kinase differentially phosphorylates p130Cas and the Cas-like protein, p105HEF1. *J Biol Chem* 1997;272:19719–30. [PubMed: 9242628]
38. Singh MK, Dadke D, Nicolas E, et al. A Novel Cas Family Member, HEPL, Regulates FAK and Cell Spreading. *Mol Biol Cell* 2008;19(4):1627–36. [PubMed: 18256281]

39. Ishino M, Ohba T, Sasaki H, Sasaki T. Molecular cloning of a cDNA encoding a phosphoprotein, Efs, which contains a Src homology 3 domain and associates with Fyn. *Oncogene* 1995;11:2331–8. [PubMed: 8570184]
40. Pylayeva Y, Gillen KM, Gerald W, Beggs HE, Reichardt LF, Giancotti FG. Ras- and PI3K-dependent breast tumorigenesis in mice and humans requires focal adhesion kinase signaling. *J Clin Invest* 2009;119(2):252–66. [PubMed: 19147981]
41. Versteeg HH, Schaffner F, Kerver M, et al. Protease-activated receptor (PAR) 2, but not PAR1, signaling promotes the development of mammary adenocarcinoma in polyoma middle T mice. *Cancer Res* 2008;68(17):7219–27. [PubMed: 18757438]
42. Cuevas BD, Winter-Vann AM, Johnson NL, Johnson GL. MEKK1 controls matrix degradation and tumor cell dissemination during metastasis of polyoma middle-T driven mammary cancer. *Oncogene* 2006;25(36):4998–5010. [PubMed: 16568086]
43. Lopez JI, Camenisch TD, Stevens MV, Sands BJ, McDonald J, Schroeder JA. CD44 attenuates metastatic invasion during breast cancer progression. *Cancer Res* 2005;65(15):6755–63. [PubMed: 16061657]
44. Liu X, Elia AEH, Law SF, Golemis EA, Farley J, Wang T. A novel ability of Smad3 to regulate proteasomal degradation of a Cas family member, HEF1. *EMBO J* 2000;19:6759–69. [PubMed: 11118211]
45. Singh M, Cowell L, Seo S, O'Neill G, Golemis E. Molecular basis for HEF1/NEDD9/Cas-L action as a multifunctional co-ordinator of invasion, apoptosis and cell cycle. *Cell Biochem Biophys* 2007;48(1):54–72. [PubMed: 17703068]
46. Law SF, O'Neill GM, Fashena SJ, Einarson MB, Golemis EA. The docking protein HEF1 is an apoptotic mediator at focal adhesion sites. *Mol Cell Biol* 2000;20:5184–95. [PubMed: 10866674]
47. Cabodi S, Tinnirello A, Di Stefano P, et al. p130Cas as a new regulator of mammary epithelial cell proliferation, survival, and HER2-neu oncogene-dependent breast tumorigenesis. *Cancer Res* 2006;66(9):4672–80. [PubMed: 16651418]
48. Honda H, Oda H, Nakamoto T, et al. Cardiovascular anomaly, impaired actin bundling and resistance to Src-induced transformation in mice lacking p130Cas. *Nat Genet* 1998;19:361–5. [PubMed: 9697697]
49. Friedman A, Perrimon N. Genetic screening for signal transduction in the era of network biology. *Cell* 2007;128(2):225–31. [PubMed: 17254958]
50. Pugacheva EN, Golemis EA. HEF1-aurora A interactions: points of dialog between the cell cycle and cell attachment signaling networks. *Cell Cycle* 2006;5(4):384–91. [PubMed: 16479169]

Figure 1. Tumor development in MMTV-PyVmT mice with *Nedd9*^{-/-} and *Nedd9*^{+/+} genotypes

A. Kaplan-Meier curves indicate significant increase in latency of tumor formation in MMTV-PyVmT;*Nedd9*^{-/-} (n=26) versus MMTV-PyVmT;*Nedd9*^{+/+} (n=21) mice (p=0.0001). **B.** Reduced tumor burden in MMTV-PyVmT;*Nedd9*^{-/-} mice (p<0.0001). **C.** Reduced number of independent tumors in MMTV-PyVmT;*Nedd9*^{-/-} (n=26) versus MMTV-PyVmT;*Nedd9*^{+/+} (n=21) mice (p=0.003). **D.** Insignificant difference in number and volume of lung metastases in MMTV-PyVmT;*Nedd9*^{-/-} and MMTV-PyVmT;*Nedd9*^{+/+} mice (p=0.569).

Figure 2. Pre-malignant lesions and mammary tumors in MMTV-PyVmT;*Nedd9*^{-/-} and MMTV-PyVmT;*Nedd9*^{+/+} mice

A, B. Early intraductal, pre-invasive lesions in young MMTV-PyVmT;*Nedd9*^{-/-} and MMTV-PyVmT;*Nedd9*^{+/+} mice at 4 and 6 weeks old, stained with H & E and Ki-67. **B.** Quantification of Ki-67 staining results for MMTV-PyVmT;*Nedd9*^{-/-} and MMTV-PyVmT;*Nedd9*^{+/+} lesions. **C.** Ki-67 staining of tumors harvested upon euthanasia.

Figure 3. Tumor-intrinsic action of *Nedd9*

A. Immunohistochemical visualization of CD31 in tumors in MMTV-PyVmT;*Nedd9*^{-/-} and MMTV-PyVmT;*Nedd9*^{+/+} mice. **B.** Quantification of **A**; no significant difference in vascularization (expressed in CD31+ blood vessels/mm²) ($p=0.5434$). **C.** Quantification of infiltration of B (CD45R), T (CD3), and macrophages (Mac-2) into tumors, expressed in cells/mm², indicates no significant differences ($p=0.3173$, 0.9818 , and 0.1047 , respectively). **D.** Representative staining for macrophage infiltration. For all panels, $n=25$ MMTV-PyVmT;*Nedd9*^{-/-} and 20 MMTV-PyVmT;*Nedd9*^{+/+} tumors.

Figure 4. Signaling pathway activation in mammary tumors

A. Signaling interactions of *Nedd9* relevant to mammary tumor development. **B, C.** Western analysis demonstrates expression and activation levels of the indicated proteins in primary tumor lysates, and quantification of relative difference in activation between *Nedd9*^{-/-} and *Nedd9*^{+/+} mice. **D.** Degree of pathway activation as a correlate of initial tumor appearance (see data in Figures 1A, 1C). The statistical trend is highly significant for time of initial detection of tumors ($p < 0.001$ for all proteins evaluated).

Figure 5. Cell invasion and signaling of MMTV-PyVmT;*Nedd9*^{-/-} versus MMTV-PyVmT;*Nedd9*^{+/+} derived mammary cells

A. Immunofluorescence showing focal adhesions (paxillin) and FAK activation (Y³⁹⁷FAK) in MMTV-PyVmT;*Nedd9*^{-/-} versus MMTV-PyVmT;*Nedd9*^{+/+} cells. Scale bar, 20 μM. **B.** Attachment of cells plated on fibronectin: values reflect traces of cell area. $P < 0.0001$ at all time points, although less prominent by 24 hours after plating. **C.** Speed of migration of individual cells plated on fibronectin. Rate of migration of PyVmT;*Nedd9*^{-/-} cells is decreased ($P = 0.029$). **D.** Quantification of cell migration calculated from wound healing assay shows significantly reduced migration in PyVmT;*Nedd9*^{-/-} cells, $P = 0.020$.

Figure 6. Nedd9 status conditions anoikis responses

A. Percent of cells positive for annexin-V after growth on poly-HEMA or on plastic. Data reflect average of three cell lines per genotype. Differences were statistically significant at 12 and 24 hours ($p < 0.002$) but not at 48 hours ($p = 0.076$) for poly-HEMA growth; a small difference was also seen in cells grown 48 hours on plastic ($p = 0.011$). **B.** Western analysis of cells grown on poly-HEMA (top) or on plastic for 48 hours. Arrow, uncleaved caspase-3 (CASP3); *, cleaved products.