NEDD9 Depletion Destabilizes Aurora A Kinase and Heightens the Efficacy of Aurora A Inhibitors: Implications for Treatment of Metastatic Solid Tumors

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Abstract

Aurora A kinase (AURKA) is overexpressed in 96% of human cancers and is considered an independent marker of poor prognosis. While the majority of tumors have elevated levels of AURKA protein, few have AURKA gene amplification, implying that posttranscriptional mechanisms regulating AURKA protein levels are significant. Here, we show that NEDD9, a known activator of AURKA, is directly involved in AURKA stability. Analysis of a comprehensive breast cancer tissue microarray revealed a tight correlation between the expression of both proteins, significantly corresponding with increased prognostic value. A decrease in AURKA, concomitant with increased ubiquitination and proteasome-dependent degradation, occurs due to depletion or knockout of NEDD9. Reexpression of wild-type NEDD9 was sufficient to rescue the observed phenomenon. Binding of NEDD9 to AURKA is critical for AURKA stabilization, as mutation of S296E was sufficient to disrupt binding and led to reduced AURKA protein levels. NEDD9 confers AURKA stability by limiting the binding of the cdh1-substrate recognition subunit of APC/C ubiquitin ligase to AURKA. Depletion of NEDD9 in tumor cells increases sensitivity to AURKA inhibitors. Combination therapy with NEDD9 short hairpin RNAs and AURKA inhibitors impairs tumor growth and distant metastasis in mice harboring xenografts of breast tumors. Collectively, our findings provide rationale for the use of AURKA inhibitors in treatment of metastatic tumors and predict the sensitivity of the patients to AURKA inhibitors based on NEDD9 expression. Cancer Res; 73(10); 3168–80. ©2013 AACR.

Introduction

The serine/threonine kinase, AURKA, is a proto-oncoprotein that is overexpressed in most cancers (1–3). High AURKA expression is strongly associated with decreased survival and is an independent prognostic marker (4). AURKA overexpression disrupts the spindle checkpoint activated by paclitaxel or nocodazole, inducing resistance to these compounds (5). Inhibition or depletion of AURKA protein may therefore improve the survival of patients resistant to paclitaxel (5). While 94% of the primary invasive mammary carcinomas have elevated AURKA protein levels (6), only 13.6% show AURKA gene amplification (1, 3). Thus, posttranscriptional mechanisms of AURKA stabilization are important in breast cancer.

AURKA is polyubiquitinated by the anaphase-promoting complex/cyclosome (APC/C) complex and targeted for degradation by the proteasome (7). APC/C-dependent degradation of AURKA requires cdh1, which acts as a substrate recognition subunit for a number of mitotic proteins, including Plk1 and cyclin B. Overexpression of cdh1 reduces AURKA levels (8), whereas cdh1 knockdown or mutation of the AURKA cdh1-binding site results in elevated AURKA expression (7–9). AURKA is ubiquitinated through the recognition of a carboxyl-terminal D-box (destruction box) and an amino-terminal A-box, specific for the destruction of AURKA (10, 11). Phosphorylation of AURKA on Ser51 in the A-box inhibits cdh1-APC/C-mediated ubiquitination and consequent AURKA degradation (9).

Cancer cells express high levels of AURKA independently of a cell cycle, which suggests that there are additional mechanisms of AURKA stabilization. Recently, a number of proteins were documented to be involved in the regulation of AURKA stability either by direct deubiquitination of AURKA (12) or through interference with AURKA ubiquitination by APC/C (PUM2, TPX2, LIMK2; refs. 13–15.)

NEDD9 is a member of a metastatic gene signature identified in breast adenocarcinomas and melanomas (16–18). NEDD9 is a cytoplasmic docking protein of the CAS family. NEDD9 regulates proliferation directly by binding to and activating AURKA (19). In nontransformed cells, activation...
of AURKA by NEDD9 in interphase is tightly controlled by a limited amount of NEDD9 in cytoplasm. Overexpression of NEDD9 leads to the activation of AURKA resulting in centrosomal amplification and aberrant mitosis (19). NEDD9 undergoes ubiquitination and proteasomal degradation by APC/C. Like typical APC/C substrates, NEDD9 has D-box motifs and cdh1 binds to a D-box located within the carboxyl-terminal domain (20, 21).

The strong link between increased AURKA expression and cancer progression has stimulated development of AURKA inhibitors for cancer therapy. PHA-680632 (22, 23), MLN8054, and MLN8237 (24, 25) are potent small-molecule inhibitors of AURKA activity. These compounds have significant antitumor activity in various animal tumor models with favorable pharmacokinetics (23). However, clinical trials with MLN8054 as a single agent failed to show tumor growth inhibition (25, 26). In the present study, using human breast cell lines and xenografts, we have identified NEDD9 as a critical regulator of AURKA protein stability and sensitivity to AURKA inhibitors. Depletion of NEDD9 via short hairpin RNAs (shRNA) decreases AURKA protein, sensitizes tumor cells to AURKA inhibitors, and eliminates metastasis in xenograft models of breast cancer. Combination therapy using NEDD9 shRNAs and AURKA inhibitors might prove to be an effective treatment strategy for solid tumors with NEDD9 overexpression.

Materials and Methods

Plasmids and reagents

shRNAs, siRNAs against human NEDD9, AURKA, and control expressed in pGIPZ or in doxycycline-inducible pTRIPZ vectors (Thermo Fisher Scientific). Lentiviral particles were prepared as previously described (26). Wild-type, Ser296Ala-A, S296/298-AAA, Ser296Glu-E, and S296/298-EE cDNAs of murine NEDD9 were subcloned into pLUTZ lentiviral vector under doxycycline-inducible promoter. pcDNA3.1-myc-Ubiquitin and pcDNA3.1-HA-NEDD9 were used for ubiquitination studies. Induction of shRNA or cDNA was done by addition of 1 μg/mL doxycycline.

Cell lines and culture conditions

The cell lines MDA-MB-231, BT-549, BT-20, ZR-75-1, MCF7, and MDA-MB-231-luc-D3H2LN (MDA-MB-231LN) expressing luciferase (Caliper Life Sciences) were purchased and authenticated by American Type Culture Collection. After infection (transfection) of shRNAs (or siRNAs), cells were selected for puromycin resistance and tested by Western blotting.

Protein stability studies

Approximately 2 × 10^6 cells were plated; 12 hours later, fresh medium containing cycloheximide (50 μg/mL) or MG132 (10 μmol/L) was added for 12 hours. At indicated time intervals, cells were lysed in protein Triton-based cell lysis buffer (19) with ubiquitin aldehyde (1–2 μmol/L), protease inhibitors (Sigma).

Cell-cycle analysis by flow cytometry

The fluorescence-activated cell-sorting (FACS) analysis was done according to a previously published protocol (19). Cell cycle distribution was analyzed by FACSCalibur equipped with Cell Quest software.

Quantitative real-time PCR

Quantitative PCR (qPCR; ref. 27) was carried out in an ABI 7500 Real-Time PCR Cycler and analyzed using Applied Biosystems SDS software.

Immunohistochemical analysis

High-density breast cancer tissue microarrays BR2082 (Supplementary Table S1) were collected with full donor consent. Immunohistochemical (IHC) procedures were done according to the manufacturer’s recommendations (US Biomax Inc.) in duplicates. Manual scoring of staining intensity [negative (0), weak (1), moderate (2+), or strong (3+)] as well as location and cell types was completed by an independent pathologist from US Biomax, Inc. Each core was scanned by the Aperio Scanning System at ×20. The total number of positive cells and the intensity of anti-NEDD9 staining were computed by Aperio ImageScope 10.1 software based on the digital images taken from each core.

Western blot procedure and antibodies

Western blotting procedures were previously described (26). Primary antibodies included anti-NEDD9 monoclonal antibody (mAb; 209; ref. 19), anti-NEDD9 (p55, custom made using NEDD9 1–394 aa as an antigen), anti-β-actin mAb, or anti-GAPDH (Sigma); anti-AURKA (BD Biosciences); anti-AURKA (AurA-N, custom made using 1–126 aa N-terminal fragment of AURKA), anti-phospho-T288 Aurora A (Cell Signaling); and anti-histone H3, and anti-phospho-Ser10 histone H3 and anti-ubiquitin (Millipore, BD Biosciences). Blots were developed by the HyGLO HRP Detection Reagent (Denville Scientific, Inc.). Bands were digitized and quantified using a digital electrophoresis documentation and image analysis system (G-box, Syngene Corp.).

Protein expression, GST pull-down, and immunoprecipitation

In vitro pull-down and immunoprecipitation protocols were previously published (19, 26). Immunoprecipitation samples were incubated with anti-AURKA (AurA-N) or anti-NEDD9 (p55), immobilized on the A/G-protein Sepharose or 4B-Glutathione agarose for GST pull-down (G&H Healthcare Life Sciences) at 4°C, washed and resolved by SDS-PAGE. Histagged cdh1 protein (Novus International, Inc.), 50 ng of recombinant AURKA and GST-HEF1 in AURKA buffer were used in the cdh1 titration pull-down.

Animal studies

NOD.Cg-Prkdc<sup>scid</sup> Il2rg<sup>−/−</sup>Wymi/SJl (NSG) immunodeficient mice were purchased from the Jackson Laboratory (stock 5557). Animals were housed in the WVU Animal Facility ( Morgantown, WV) under pathogen-free conditions; protocol approved by the Institutional Animal Care and Use Committee. Primary tumor and organs with metastases were collected.
processed, and analyzed by the WVU Department of Pathology Tissue Bank Core Facility.

**Animal bioluminescence imaging**

Mice were injected with luciferase-expressing MDA-MB-231LN cells and imaged weekly for quantitative evaluation of tumor growth and dissemination. About 150 mg/kg d-luciferin (Caliper Life Sciences) was injected into the peritoneum. Images were obtained using the IVIS Lumina-II Imaging System and Living Image-4.0 software.

1. Mammary fat pad injections: For animal studies, cells were grown, trypsinized, resuspended in DPBS (1 x 10^7 cells/mL), and 0.1 mL was injected into the fourth inguinal mammary gland of female mice 6 to 8 weeks of age and followed by bioluminescence imaging (BLI) up to 6 weeks.

2. Tail vein injections: Males were intravenously injected with 1 x 10^7 cells and followed by BLI once a week for 2 to 3 weeks total. Total radiance of lungs was calculated at each time point in control and treated animals. Lungs were imaged, fixed in formalin at the end point of study, and analyzed for number and size of metastases by a pathologist.

**Tumor volume measurement**

Tumor size was assessed by Vevo2100 Micro-Ultrasound System. A 40 or 50 mHz transducer was used, depending on the tumor size, and a 3-dimensional image (3D) was acquired with 0.051 mm between images. Using the integrated software, the images were reconstructed to create a 3D image of the tumor.

**AURKA inhibitors application**

**Cell line studies.** Cells were treated with MLN8054 (0–100 nmol/L) or PHA-680632 (0–400 nmol/L) inhibitors (Selleckchem) for 2 to 12 hours, disrupted in protein Triton-based cell lysis buffer (19) processed for Western blotting or immunofluorescence staining.

**Xenograft studies.** Compound administration began (i) when primary tumors reached 150 to 200 mm^3 in female mice or (ii) 24 hours postintravenous injection of tumor cells in male mice. MLN8237 is an improved analog of MLN8054 compound with increased stability suitable for in vivo studies. MLN8237 was dissolved in 10% 2-hydroxypropyl-β-cyclodextrin, 1% sodium bicarbonate in water. About 20 mg/kg/dose was administered via oral gavage twice daily for 4 days/week for 2 weeks. MLN8237 was tested against a placebo control consisting of drug vehicle.

**Statistical analysis**

Unpaired t test, nonlinear regression, or 1- or 2-way ANOVA with Tukey’s multiple comparisons were used for statistical analysis of the results. Experimental values were reported as SEM. Differences in mean values were considered significant at P < 0.05. Rates of tumor growth were established by linear regression of the bioluminescence data with time and cohort membership as covariates. Statistical calculations were conducted using the GraphPad InStat software package.

**Results**

**Increased NEDD9 expression tightly correlates with expression of AURKA protein in breast cancer**

AURKA and NEDD9 are independently overexpressed in many human cancers (1–3, 16–18). We have previously shown that NEDD9 binds to and activates AURKA in cancer cells (19), and expression of AURKA alone was found to be an independent prognostic marker of poor survival. To determine whether the expression of both proteins could facilitate the diagnosis of certain types or stages of breast cancer, we conducted IHC staining for NEDD9 and AURKA in 120 cases of breast cancer. Cases screened from a tissue microarray consisted of 4 groups of progressive disease stages: (i) normal tissue, (ii) intraductal carcinoma (IC), (iii) invasive ductal carcinoma (IDC), and (iv) metastatic IDC (MIDC; Supplementary Table S1). Representative images of IHC staining for each group are shown in Fig. 1A. Statistical analysis of staining intensity suggests that NEDD9 and AURKA expression positively correlate. The lowest expression and intensity of either protein was found in normal tissue, whereas a 10- to 20-fold increase in expression was observed in tumor samples (Fig. 1B). Significant correlation between NEDD9 and AURKA expression was noted for all 4 evaluated tissue types (Fig. 1C). The Spearman correlation coefficients for the normal, IC, IDC, and MIDC groups are 0.85, 0.63, 0.67, and 0.59, respectively, indicating a positive correlation (Fig. 1C). A random forest fit of NEDD9 and AURKA positivity staining, which achieved an out-of-bag error rate of 0.508, indicates that by using NEDD9 and AURKA positivity scores, one can double the predictive power over chance (Fig. 1D).

**Depletion of NEDD9 leads to dramatic decrease of AURKA protein in cells lines and animal models**

The expression profiles of AURKA and NEDD9 in a panel of human breast cancer cell lines followed a pattern similar to that observed in the tissue microarray analysis. Invasive MDA-MB-231 (or highly invasive lymph node–derived MDA-MB-231LN), MDA-MB-453, andZR-75-1 cell lines had the highest levels of expression of NEDD9 and AURKA, followed by BT-549, and noninvasive MCF7 and BT-20 lines (Fig. 2A). We next evaluated AURKA protein levels in mouse embryonic fibroblasts derived from NEDD9 knockout (KO) and wild-type (WT) animals. AURKA expression levels were reduced in KO cells compared with WT cells (Fig. 2B). Similar results were obtained by IHC analysis of tissue sections (Fig. 2C), suggesting that maximal expression of AURKA is dependent on NEDD9.

Depletion of NEDD9 by 2 different shRNAs or siRNAs reduced the levels of AURKA protein by 60% to 80% (Fig. 2D; Supplementary Fig. S1A). To determine whether the reduced levels of AURKA were due to transcriptional mechanisms or nonspecific siRNA depletion, we carried out qRT-PCR analysis for NEDD9 and AURKA. The NEDD9-targeting siRNAs did not affect the levels of AURKA mRNA (Supplementary Fig. S1B), indicating that NEDD9 regulates AURKA at the protein level. Moreover, we were able to restore AURKA protein levels
in shNEDD9 cells via reexpression of doxycycline-inducible WT-NEDD9 cDNA (Fig. 2E).

Protein levels of NEDD9 and AURKA are tightly regulated during the cell cycle (28); therefore, we examined the effects of NEDD9 depletion on the cell cycle. FACS analysis of cells treated with siRNA targeting NEDD9 did not show significant difference in cell-cycle distribution when compared with siCon (Supplementary Fig. S1C). These results indicate that the decrease in AURKA protein level is NEDD9-dependent and is posttranscriptionally regulated.

**NEDD9 regulates the stability of AURKA**

To further evaluate how NEDD9 governs AURKA expression, we examined AURKA levels in shCon- and shNEDD9-MDA-MB-231LN cells treated with the protein synthesis inhibitor cycloheximide. Cycloheximide treatment led to an abrupt decrease in the amount of AURKA in shNEDD9 cells during the first 3 hours (Fig. 3A). shCon cells had elevated AURKA levels for 6 hours and followed NEDD9 protein decay dynamics (Fig. 3A). The half-life of AURKA was 6 and 3 hours in shCon and shNEDD9, respectively (Fig. 3B). The delayed decrease in...
AURKA protein levels in control cells compared with shNEDD9 indicates that AURKA protein stability is dependent on NEDD9.

**Decreased AURKA protein in NEDD9-deficient cells is caused by enhanced proteasome-dependent degradation**

NEDD9 and AURKA undergo ubiquitination and proteasomal degradation in a cell-cycle-dependent manner (9, 20). To test whether the decrease in AURKA protein levels is associated with increased proteasome-based degradation, cell lines with depleted NEDD9 were treated with the proteasome inhibitor MG132 (Fig. 3C). Inhibition of proteasomal activity restored levels of AURKA in shNEDD9 cells to that of control cells (Fig. 3C and D), suggesting that NEDD9 protects AURKA from ubiquitination- and proteasome-dependent degradation. To directly test this, AURKA was immunoprecipitated from shNEDD9 and control cells and analyzed by immunoblotting with anti-AURKA and antiubiquitin antibodies. Depletion of NEDD9 increased the amount of ubiquitinated AURKA (Fig. 3E and F). Similar results were obtained with original MDA-MB-231 cells. Moreover, reexpression of wild-type NEDD9 was able to rescue this phenotype and decrease ubiquitination of AURKA (Supplementary Fig. S1E and S1F).

NEDD9-dependent decrease in AURKA ubiquitination could be caused by steric hindrance of bound NEDD9 or by titration of ubiquitination machinery components, as both proteins use the APC/C–cdh1 complex (8, 20). To distinguish between these 2 possibilities, the levels of other APC/C–cdh1 targets, including Plk1 and Cdk1, in shNEDD9 cells were evaluated by immunoblotting. No difference in Plk1 and Cdk1 expression to shCon, normalized by GAPDH. Student one-tailed t test: *P = 0.0094; **P = 0.0024 (shCon/shN1 or/shN2) and no significant difference (ns: shN1/shN2) in MCF7, **P = 0.0031; *P = 0.0094 (shCon/shN1 or/shN2) and ns (shN1/shN2) in MDA-MB-231. E, WB analysis and quantification of AURKA and NEDD9 expression in shNEDD9 and shCon cells transfected with WT-NEDD9 or control-RFP (red fluorescent protein) cDNA; n = 3, percentage of AURKA expression to shCon, normalized by GAPDH. Student one-tailed t test: *P = 0.0186; **P = 0.0012 (shCon/shN1 or/shN2), ns (shN1/shN2 or shN1/NEDD9 and shN2/NEDD9 reexpression).
Figure 3. NEDD9 regulates stability of AURKA through inhibition of proteasome-dependent degradation. A, Western blot analysis of AURKA and NEDD9 expression in shCon-, shNEDD9-MDA-MB-231LN cells treated with cycloheximide. Expression was normalized by α-tubulin. B, quantification of AURKA as in A, n = 3, fold of change of AURKA expression to time point 0 hours, ±SEM, one-way ANOVA: * * * P < 0.0001; * * P = 0.005 (shCon/shN1 or /shN2) at each time point (except 0 hour). C, Western blot analysis of AURKA, NEDD9 in shCon, shNEDD9 cells treated with MG132 or vehicle. D, quantification of AURKA expression as in C (+MG132), n = 3, fold of change to shCon, ±SEM. Student t test: * * * P = 0.0086; * * P = 0.0084 (shCon/shN1 or /shN2). E, Western blot analysis of AURKA ubiquitination in WCL and immunoprecipitation (IP) from shCon or shNEDD9 cells transfected with pcDNA3-Myc-Ubiquitin, with MG132. F, quantification of AURKA ubiquitination as in E, IP, n = 3, fold of change to shCon, ±SEM. Student t test: * * * P = 0.0086; * * P = 0.0084 (shCon/shN1 or /shN2). G, WB with anti-AURKA, anti-NEDD9, and anti-cdh1 antibodies. H, quantification of AURKA using the following formula (AURKA-IP/AURKA-total) normalized to NEDD9 pull-down as in G, n = 3, plotted as AURKA ratio ± SEM, * * P < 0.05.
N-terminal domain of AURKA containing the A-box motif (19), which is required for cdh1 binding and ubiquitination by APC/C (7, 8). To test this hypothesis, we used recombinant NEDD9, cdh1, and AURKA in in vitro GST pull-down assay and examined whether the presence of NEDD9 imposes its inhibitory action on cdh1 directly. We confirmed that NEDD9 was able to bind AURKA in vitro in the presence of excess cdh1 and titrated cdh1 from the complex with AURKA in a concentration-dependent manner (Fig. 3G and H). Thus, the presence of NEDD9 potentially increases AURKA protein levels by protecting AURKA from binding cdh1 resulting in reduced AURKA ubiquitination and subsequent proteasomal degradation.

**NEDD9 binding to AURKA is necessary for protein stabilization**

Phosphorylation of NEDD9 at S296 and S298 by AURKA impedes formation of the NEDD9/AURKA complex (19). To determine the impact of NEDD9 binding on AURKA protein levels, individual and dual phosphorylation null S296A-(A), S296A/S298A-(AA) and mimetic S296E-(E), S296E/S298E-(EE) forms of NEDD9 were generated. Indicated mutants were transfected in HEK293T cells followed by immunoprecipitation of AURKA (Fig. 4A). Immunoprecipitation analysis showed a reduction in binding to AURKA by the NEDD9 phosphomimetic (E, EE) mutants, whereas phosphorylation null (A, AA) NEDD9 mutants showed increase in AURKA binding (Fig. 4A and B), in agreement with our previously published observations (19). The increase in binding by phosphorylation-null mutants in this setting is expected because of overexpression of NEDD9 in HEK293T cells and inability of AURKA/NEDD9-9A complex to dissociate. To test the ability of these mutants to rescue the levels of AURKA in shNEDD9 cells, the 2 NEDD9 mutants (AA, EE) were overexpressed in an inducible manner and AURKA levels were measured by Western blotting (Fig. 4C and D). Reexpression of the AA mutant was sufficient to restore the levels of AURKA protein, meanwhile, the EE mutant failed to restore the levels of AURKA to control levels due to its inability to bind AURKA. Therefore, binding of NEDD9 to AURKA is necessary to stabilize AURKA.

**NEDD9 binding to AURKA decreases the efficacy of AURKA inhibitors in vitro and in vivo xenografts**

Because of the structural proximity of the ATP-binding pocket to the NEDD9-binding domain on AURKA (23), we hypothesized that NEDD9-AURKA binding could potentially interfere with binding of AURKA inhibitors, in addition to preventing cdh1 binding. MLN8054 and PHA-680632 are ATP-competitive AURKA inhibitors that are currently in phase II clinical trials that have shown some efficacy against hematopoietic malignancies but have minimal effects against solid tumors (29–31). To evaluate the impact of NEDD9 expression on therapeutic outcome, NEDD9 was knocked down in breast cancer cell lines with high NEDD9 expression before treatment with PHA-680632 or MLN8054. NEDD9 depletion increased the efficacy of both inhibitors (Fig. 5A), decreasing the IC50 of PHA-680632 from 150 nmol/L in control cells to 50 to 100 nmol/L in shNEDD9 cells (Fig. 5B), as determined by the amount of active pThr288-AURKA. Similar results were obtained with MLN8054, where the IC50 value decreased from 200 nmol/L (shCon) to 20 nmol/L (shNEDD9; Fig. 5C and D). Inhibition of AURKA function was further confirmed by analysis of histone H3 phosphorylation in treated shCon and shNEDD9 cells. Inhibition of AURKA leads to accumulation of cells in mitosis characterized by phosphorylation of histone H3 at Ser10 (Fig. 5B and D and Supplementary Fig. S2E). The concentration of PHA-680632 was limited to 400 nmol/L to avoid targeting AURKB, which might lead to decrease in phosphorylation of histone H3.

**NEDD9 depletion alone or in combination with AURKA inhibitors reduces tumor burden and lung metastasis**

To examine the validity of our findings in in vivo xenograft models of human breast cancer, we used MDA-MB-231LN cells and shRNAs targeting NEDD9 or control. Tumor cells were injected into the mammary fat pad of NSG female mice and the tumor growth was assessed by BLI (Fig. 6A and B). The original MDA-MB-231LN cell line was tested and showed similar NEDD9 expression, tumor growth, and metastasis kinetics when compared with MDA-MB-231LN-shCon cells (Fig. 6A and B and Supplementary Fig. S1F). On the basis of these results, we have concluded that the MDA-MB-231LN-shCon cell line is a proper control and we used it in the subsequent experiments. Depletion of NEDD9 alone reduced tumor burden by 15% to 20% (Fig. 6B) and reduced the number of metastases in lungs by 25% to 30% (Fig. 6D and E shCon-V, shN2-V and Supplementary Fig. S2A).

Next, we combined shNEDD9 and AURKA inhibitor (MLN8237, a more stable analog of MLN8054) to test whether the combination will increase the efficacy of MLN8237 against primary tumor and metastasis (Fig. 6C–E). Treatment was initiated when primary tumor volume reached 150 to 200 mm3, based on ultrasound measurements in each cohort (30). Representative images and quantification of tumor volume is shown in Supplementary Fig. S2B and S2C. Difference in the rates of tumor growth among the 4 groups was assessed by BLI and pathology measurements using linear regression analysis with time, cell line, and treatment as covariates. Application of MLN8237 alone did not lead to a decrease in primary tumor growth, but in combination with shNEDD9, efficacy was improved 2-fold (Fig. 6C). Surprisingly, treatment with MLN8237 alone significantly decreased the number of lung metastases with a 2-fold greater response in shNEDD9-expressing cells (Fig. 6D and E; Supplementary Fig. S2D).

Next, we used intravenous injection of breast cancer cells in NSG male mice to determine the impact of AURKA inhibitor on colonization by circulating tumor cells. On the basis of BLI data (Fig. 7A–C) and study endpoint pathology reports on dissected lungs (Supplementary Fig. S2F), shNEDD9-expressing cells were extremely sensitive to MLN8237 and were not capable of initiating tumor growth in lungs when compared with vehicle-treated cells. In summary, depletion of NEDD9 sensitizes human xenografted tumors and circulating tumor cells to AURKA inhibitors and eliminates metastasis to the lungs. Collectively, our data suggest a model (Fig. 7D) in which overexpression of NEDD9 renders AURKA less...
susceptible to Cdh1-APC–mediated ubiquitination and binding of small-molecule ATP-competitive inhibitors, thus protecting it from degradation and drug applications.

Discussion

Recent studies corroborate overexpression of NEDD9 specifically with breast cancer and melanoma metastasis (16–18).

Our data indicate that NEDD9 expression levels correlate positively with oncogenic AURKA expression and activation. Both proteins were correlated with pathologic parameters in human breast cancer cell lines and breast cancer patient samples. Moreover, achieving 80% accuracy for predicting cancer stage invasiveness is possible when screening for expression of both proteins.
Figure 5. NEDD9 binding to AURKA decreases the efficacy of AURKA inhibitors in vitro. A, Western blot analysis of AURKA, PhT288-AURKA, NEDD9 in shCon or shNEDD9-MDA-MB-231LN cells treated with PHA-680632. B, quantification of PhT288-AURKA expression as in A, n = 3, normalized to total AURKA and plotted as relative intensity units (RIU) ± SEM. Two-way ANOVA: P < 0.0001, (shCon/shN1 or shN2), P = 0.0013 (shN1/shN2). Quantification of phS10-Histone H3 (IF), n = 3, 1000 cells/treatment, P = 0.006, P = 0.0067 (shCon/shN1 or shN2), no significant difference (shN1/shN2). C. Western blot analysis of total AURKA, PhT288-AURKA, NEDD9 expression in cells treated with increasing concentrations of MLN8054. D, quantification as in B for the Western blots shown in C. P < 0.0001, (shCon/shN1 or shN2), P = 0.0041 (shN1/shN2). Quantification of immunofluorescence shown in Supplementary Fig. S2E, n = 3, 1,000 cells/treatment, P = 0.0053, P = 0.0086 (shN1/shN2), no significant difference (shN1/shN2).
AURKA inhibitors have recently entered phase II clinical trials for cancer treatment. However, the best response achieved in advanced tumors is disease stabilization, as tumor regression has not been reported (29–32). The knowledge of the molecular factors that influence AURKA stability and, therefore, sensitivity and resistance to AURKA inhibitors remains limited. With a few exceptions, such as TPX2 and PUM2 (13, 14), the role of AURKA activators in the stability of AURKA and their impact on sensitivity to AURKA inhibitors is unknown.

We show here that NEDD9 is a critical component in AURKA activation and stability. Furthermore, the molecular mechanism by which NEDD9/AURKA signaling functions to increase breast tumor cell resistance to AURKA inhibitors has been elucidated. Overexpression of NEDD9 in breast cancer cells prevents proteolytic degradation of AURKA and results in upregulation of AURKA protein level. AURKA activation correlates with protein stabilization, which in turn directly depends upon APC/C complex and its substrate recognition subunit, cdh1. Direct binding of NEDD9 to AURKA hampers the ability of the APC/C complex to ubiquitinate AURKA by preventing cdh1 binding. A NEDD9-dependent increase in AURKA protein and activity is critical for G2–M transition. Interestingly, the majority of proteins activating and/or stabilizing AURKA reside in the nucleus (Bora, TPX2, Ajuba, etc.) including phosphorylation by AURKA for binding (33–36). Nevertheless, a NEDD9-driven increase in the total amount of AURKA would be less noticeable in mitosis due to the inactivity of cdh1 (8). The excess of NEDD9 protein might promote the binding of known AURKA partners such as Ajuba, TPX2, and Bora (33, 34) leading to increased loading of AURKA on microtubules and Plk1 activity (37) and cancer progression. Direct binding of NEDD9 at the N-
Figure 7. Combination of AURKA Inhibitors with depletion of NEDD9 reduces colonization potential of human circulating tumor cells in lungs. A, representative images of BLI; mice intravenously injected with MDA-MB-231LN cells expressing shCon or shNEDD9 (shN2) and treated with MLN8237 (M) or vehicle (V). B, quantification of BLI data as in A, 3 independent experiments, n = 6/group, plotted as fold growth of BLI mean radiance ± SEM. Two-way ANOVA: P < 0.0001 (shCon-V/shCon-M), (shN2-V/shN2-M), (shCon-V/shN2-V); P = 0.0145 (shCon-M/shN2-M). C, quantification of BLI as in A, dissected lungs; 3 independent experiments, n = 6/group, mean photon flux ± SEM. One-tailed Student t test: P < 0.0001 (shCon-V/shCon-M), (shN2-V/shN2-M), (shN2-V/shN2-M), (shN2-V/shN2-M); P = 0.009 (shCon-V/shN2-V), P = 0.0335 (shCon-M/shN-M). D, model of NEDD9 action on AURKA stability and inhibitors competitive binding (I/III). Under normal physiologic conditions with low NEDD9 expression, AURKA is not solely bound to NEDD9 and gets targeted by cdh1 and AURKA inhibitors (II/IV). Overexpression of NEDD9 leads to sequestering of AURKA by NEDD9 and limits cdh1 binding, thus stabilizing AURKA and hampers the ability AURKA inhibitors to access ATP packet.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Zhou H, Kuang J, Zhong L, Kuol WL, Sahin A, et al. Tumour amplified kinase STK15/BAK generates a stabilizing loop to regulate the levels of active AURKA. The abundance of NEDD9 in epithelial cancers and dephosphorylation by PP2A (21) creates a constant supply of unphosphorylated NEDD9 that would stabilize and activate AURKA. We found that depletion of NEDD9 reduces tumor cell proliferation and lung metastases of orthotopic human tumor xenografts. Finally, we have established that treatment with AURKA inhibitors is particularly efficient against metastasis. In combination with shNEDD9 RNAs, MLN8237 abolishes lung metastases from orthotopic xenograft models as well as in lung colonization assays. No significant changes in animal weight and no apparent toxicity were noticed, suggesting a favorable toxicity profile.

The correlation of AURKA and NEDD9 expression in cancer patient biopsies could be critical for diagnostic purposes. It could potentially be used to predict the sensitivity of these patients to AURKA inhibitors. In addition, our results advocate the development and investigation of new NEDD9-targeting compounds as a novel therapeutic strategy against metastatic breast cancer.

NEDD9 Stabilizes AURKA

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Ice, M. Calyp, E. Eddy, A.V. Ivanov, E.N. Pugacheva

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