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N-α-Acetyltransferase 10 Protein Suppresses Cancer Cell Metastasis by Binding PIX Proteins and Inhibiting Cdc42/Rac1 Activity

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SUMMARY

***N*-α-acetyltransferase 10 protein, Naa10p, is an *N*-acetyltransferase known to be involved in cell cycle control. We found that Naa10p was expressed lower in varieties of malignancies with lymph node metastasis compared with non-lymph node metastasis. Higher Naa10p expression correlates the survival of lung cancer patients. Naa10p significantly suppressed migration, tumor growth, and metastasis independent of its enzymatic activity. Instead, Naa10p binds to the GIT-binding domain of PIX, thereby preventing the formation of the GIT-PIX-Paxillin complex, resulting in reduced intrinsic Cdc42/Rac1 activity and decreased cell migration. Forced expression of PIX in Naa10-transfected tumor cells restored the migration and metastasis ability. We suggest that Naa10p functions as a tumor metastasis suppressor by disrupting the migratory complex, PIX-GIT- Paxillin, in cancer cells.**

INTRODUCTION

N-α-acetyltransferase 10 (Naa10, also known as ARD1) protein is the catalytic subunit of the major N-terminal acetyltransferase (NAT) complex, NatA, consisting of Naa10p and human Naa15p (also known as NATH). Together, they mediate the N-terminal acetylation of nascent polypeptides that emerge from ribosomes after translation (Arnesen et al., 2009). The specificity of Naa10p is highly conserved from yeasts to humans (Arnesen et al., 2005, 2009; Park and Szostak, 1992; Whiteway and Szostak, 1985). *Ard1*-Δ yeast mutants and Naa10p knockdown cells show a similar defect in cell growth (Fisher et al., 2005; Lim et al., 2006; Whiteway and Szostak, 1985). Recently, Naa10p expression was reported in breast, cervical, thyroid, and colorectal

Significance

The role of Naa10p in human cancers is elusive. In the present study, we found that elevated Naa10p levels are associated with less metastasis to lymph nodes and better prognosis of cancer patients. Naa10p inhibits cell migration/invasion through a previously undescribed mechanism of binding to the PIX protein, thereby preventing its translocation to the membrane and reducing subsequent Cdc42/Rac1 activity. An increased expression of PIX reversed the suppressive effect of Naa10p on cell migration and tumor metastasis. Our study not only highlights the potential of Naa10p as a therapeutic target for tumor metastasis, but also identifies Naa10p as a regulatory component of the migration complex PIX-GIT-Paxillin.

cancer (Fluge et al., 2002; Ren et al., 2008; Yu et al., 2009). However, the correlation between Naa10p and important clinical parameters, as well as the exact action of Naa10p in cancer cells remain unclear.

During cell migration, actin filaments organize into a branched network at the leading edge, and the filaments undergo rapid and coordinated assembly and/or disassembly to drive the edge forward (Pollard and Borisy, 2003). The concerted regulation of these events is mediated by a complex temporal and spatial interplay of Rho GTPases and their regulatory proteins, including guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs) (Gulli and Peter, 2001). The p21-activated kinase (PAK)-interacting exchange factor (PIX) family members have been shown to act as GEFs mediating the activation of Rac1 and Cdc42. In addition, PIX proteins can serve as scaffold proteins by binding to Paxillin and G protein-coupled receptor kinase-interacting (GIT) proteins, the GAP for Arf GTPases, resulting in translocation of the GIT-PIX-Paxillin complex to a focal adhesion complex and membrane ruffles (Botrugno et al., 2006; Hoefen and Berk, 2006; Webb et al., 2002). The PIX complex serves as a multifunctional protein binding platform that plays an important role in the control of cell shape, polarity, and focal adhesion turnover (Frank and Hansen, 2008; Hoefen and Berk, 2006; Jones and Katan, 2007; Zhang et al., 2005). PIX proteins were found to be highly expressed in glioma (Yokota et al., 2006) and breast cancer (Ahn et al., 2003). Both PIX and GIT were found to increase the migration potentials of a variety of cancer cells (Lee et al., 2005; Manabe et al., 2002). Recently, the GEF activity of PIX was found to be regulated by phosphorylation at specific sites and its binding interactions (Chahdi et al., 2005; Shin et al., 2002, 2004). Binding of PAK and GTP-bound Cdc42 stimulates αPIX GEF activity (Baird et al., 2005; Feng et al., 2004), while binding of 14-3-3β inhibits βPIX GEF activity (Chahdi and Sorokin, 2008). Nonetheless, the regulation of PIX localization and the formation of the PIX-GIT-Paxillin complex remain unknown.

Here, we investigated whether Naa10p expressed differentially in tumors of different progression status and the involvement of Naa10p in the regulation of PIX-GIT complex formation and function.

RESULTS

Expression of Naa10p Inversely Correlates with Lymph Node Metastasis and Survival of Cancer Patients

To elucidate the clinical relevance of Naa10p in cancer patients, we analyzed a cohort of 318 lung cancer specimens using immunohistochemical analysis (IHC) with Naa10p-specific antibody. The antibody specificity was defined and is shown in Figure S1A available online. Naa10p expression was detected in normal lung epithelium (Figure 1A), lung adenocarcinomas (Figures 1B and 1C), and in squamous cell carcinomas (Figures 1D and 1E). Naa10p was found to be expressed predominantly in the cytosol, and rarely in the nucleus. Naa10p expression was inversely correlated with lymph node metastasis (Table 1). Semi-quantitative analysis by immunoblotting of an independent cohort of lung adenocarcinoma (N = 89) also confirms this correlation (Figure 1F). To study the significance of Naa10p in lymph node metastasis, we collected 15 sets of individually matched samples from primary tumors and lymph node metastases. Strikingly, in 13 out of 15 patients, the Naa10p levels were significantly reduced in the lymph node metastatic tumors compared with primary lung tumors (Figures 1G and 1H, $p < 0.01$). Notably, the early stage tumor without lymph node involvement had the highest level of Naa10p expression (case 1 in Figure 1G). Similar results were also found in breast (n = 53) and gastric cancer tissues (n = 92) by comparing patients with or without lymph node metastasis (Figures S1E to S1G). Naa10p expression was significantly correlated with both relapse-free survival and overall survival of lung cancer patients (Figure 1I; Figures S1B–S1D). Taken together, the above clinical data indicate that down-regulation of Naa10p is a critical event in tumor progression.

Naa10p Suppresses Mobility of Cancer Cells

Our clinical findings suggest that Naa10p may play a role in the invasiveness and metastatic ability of tumor cells. We next evaluated the function of Naa10p in cell migration, which is the most fundamental step of tumor invasion and metastasis. siRNAs knockdown of Naa10p significantly increased cell motility in lung cancer cell lines with limited transwell migration ability (CL1-0, H661, PC14, and H1435) (Figure 2A; Figure S2A). To exclude the possibility of off-target effects, we reintroduced Naa10p in to the cells, bringing the migration level back to normal (Figure 2B). In comparison, overexpression of Naa10p significantly decreased cell migration in highly migratory lung cancer cell lines CL1-5, H1355, and A549 (Figure 2C). These results were also confirmed in a series of breast, ovarian, and gastric cancer cell lines (Figures S2B). Similar results were obtained in cell tracking assays (Figure S2C). Naa10p affected the invasion ability and migration of cancer cells to a similar extent (Figure S2D). However, we did not detect any significant difference in matrix metalloproteinase levels among these cells (data not shown). Thus, the effect of Naa10p on cell invasiveness was largely attributable to migration inhibition. In addition, manipulation of Naa10p expression had no significant effect on cell proliferation (Figure S2E). Furthermore, the distribution of focal adhesions was altered in the Naa10p-knockdown AGS cells as detected by paxillin-specific antibody. The control AGS cells displayed an immotile phenotype with random distribution of paxillin around the cell, while most of the Naa10p-knockdown cells displayed one or more pseudopodia-like membrane protrusions, which may explain the increase in migration ability. The control CL1-5 cells had most of their focal adhesions in the puncta of membrane protrusions, while Naa10p-expressing cells displayed a more random distribution (Figure 2D). Taken together, these results indicate Naa10p plays important roles in cell migration.

Acetyltransferase Activity of Naa10p Is Not Necessary for Regulating Cell Migration

Naa10p exerts its acetyltransferase activity through collaboration with Naa15p (Arnesen et al., 2005). To determine whether Naa10p affects cell migration in a Naa15p-dependent manner, we cotransfected Naa10p and Naa15p into A549 cells and measured their relative migration abilities. Comparing to the known cooperative role of Naa10p and Naa15p in other cellular functions (Arnesen et al., 2005; Asaumi et al., 2005;

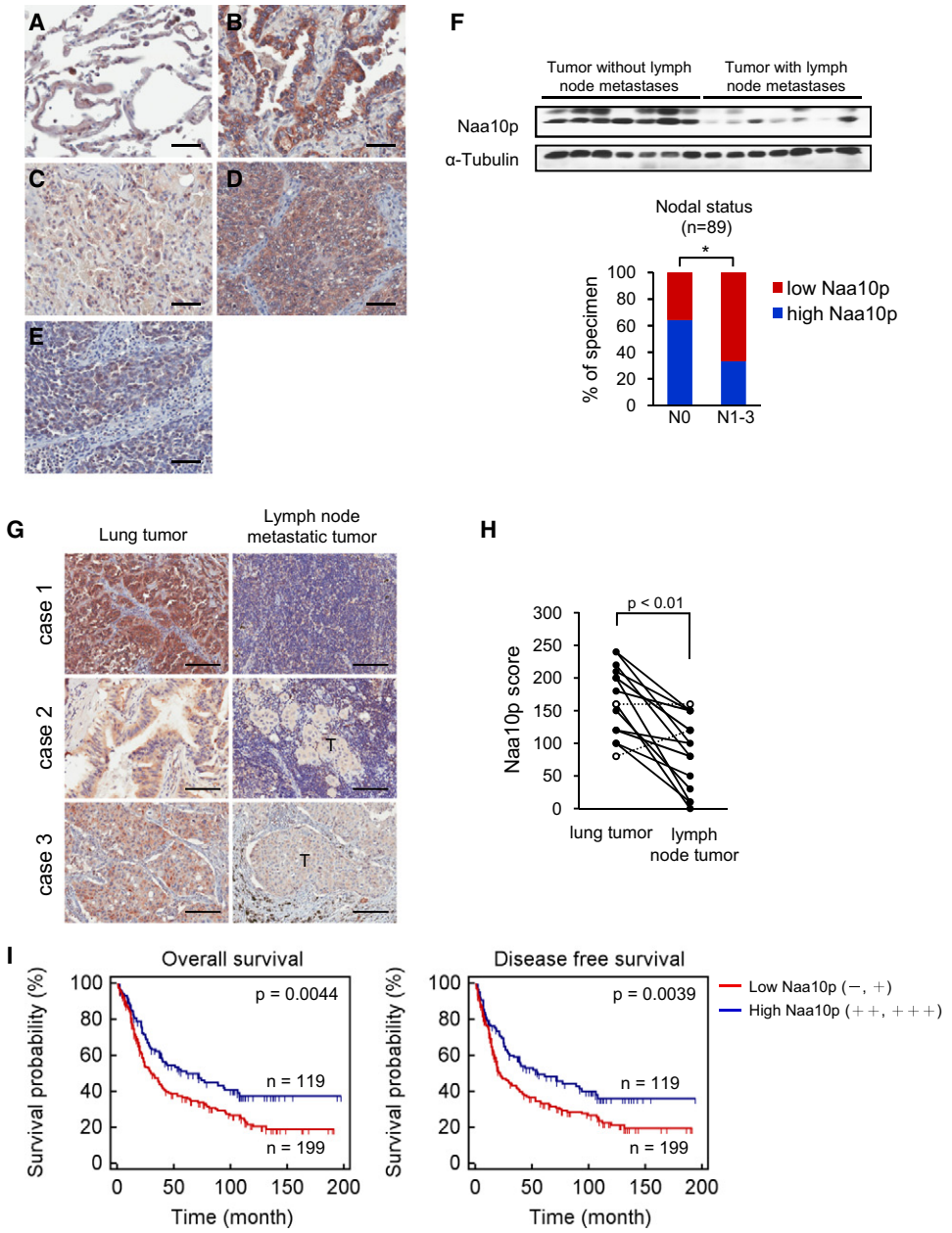

Figure 1. Naa10p Is Expressed in Cancers and Inversely Correlates with Lymph Node Metastasis and Survival of Cancer Patients

(A–E) Naa10p levels in representative normal and tumor tissues. Immunohistochemistry using the rabbit anti-Naa10p antibody (GB-10511, Genesis Biotech) of normal lung alveolar tissue (A), lung adenocarcinoma tissues (B and C) and lung squamous cell carcinoma tissues (D and E). (B) and (D) are examples of high Naa10p expression; (C) and (E) are example of low Naa10p expression. Scale bars, 50 μm in (A)–(E).

(F) Correlation between Naa10p expression levels and lymph node metastasis status. A total of 89 lung adenocarcinoma specimens were analyzed by immunoblotting. After normalization with β-actin, the average expression level was recognized in arbitrary units (AU) 1. Low Naa10p expression (AU < 0.45) is depicted as red columns, whereas high Naa10p expression (AU > 0.45) is depicted as blue columns. *$p < 0.05$.

(G) Representative microphotographs of immunohistochemical staining of Naa10p in matched specimens of primary lung tumor and lymph node metastases. T, tumor cells in lymph node. Scale bars, 100 μm.

(H) Plot representation of scores according to cytoplasmic immunohistochemical expression of Naa10p in primary lung tumors related to the lymph node metastases. The scores are calculated by intensity × percentage of stained cells.

(I) Kaplan-Meier plot of overall and disease-free survival of 318 patients with lung carcinomas stratified by Naa10p expression level. A log rank test was used to show differences between groups.

See also Figure S1.

Table 1. Clinicopathologic Characteristics of Patients with Low and High Expression of Naa10p in 318 Lung Cancer Patients

Characteristic	Low Expression (score 0–1), N = 199	High Expression (score 2–3), N = 119	p Value
Age (mean ± SD) (in years)	61.76 ± 11.36	61.77 ± 10.99	0.99[a]
Sex, no. of patients			
—Male	120	67	0.48[b]
—Female	79	52	
Stage,[c] no. of patients			
—I-II	107	78	0.04[b]
—III-IV	92	41	
Tumor status,[c] no. of patients			
—T1-T2	161	101	0.37[b]
—T3-T4	38	18	
Lymph nodal status,[c] no. of patients			
—N0	89	69	0.02[b]
—N1-N3	110	50	
Metastasis,[c] no. of patients			
—M0	164	98	0.99[b]
—M1	35	21	
Histology, no. of patients			
—Adenocarcinoma	131	84	0.38[b]
—Nonadenocarcinoma	68	35	
Median survival (in months)	61.2	29.8	0.0044[d]

[a] p value was measured with student's t test.
[b] p values were derived with Pearson chi-square tests.
[c] The tumor stage, tumor status, lymph node status, and metastasis were classified according to the international system for staging lung cancer.
[d] p values was derived with log rank test. All statistical tests are two sided. SD, standard deviation.

Lim et al., 2006), Naa10p-mediated suppression of migration was not accelerated in the presence of Naa15p (Figure 2E, column 4 versus column 2). To confirm whether the acetylation activity of Naa10p was involved in regulating cell mobility, we generated a mutant Naa10p construct, Naa10p-R82A, which had lost its ability to associate with acetyl-CoA, and therefore exhibited low acetyltransferase activity (Asaumi et al., 2005) as confirmed by [^{3}H]-acetate incorporation assay using PCNP, a known Naa10p N-acetylation target (Arnesen et al., 2009), as substrate (Figure S2F). Interestingly, no difference in the migration ability was detectable between Naa10p- and Naa10p-R82A-transfected A549 cells (Figure 2E). Similarly, overexpression or shRNA inhibition of Naa11p, a homolog of Naa10p that displays similar acetyltransferase activity, did not affect cell migration (Figures 2F and 2G). Taken together, these results suggest that Naa10p regulates cell migration through a mechanism independent of Naa15p and its intrinsic acetyltransferase activity.

Suppression of Tumor Growth and Metastasis by Naa10p

We next examined the in vivo effects of Naa10p expression on tumor growth and metastasis. Control CL1-5 cells (CL1-5/vec) subcutaneously injected into NOD-SCID mice formed large tumors after 4 weeks. In contrast, mice injected with CL1-5 cells ectopically expressing Naa10p (CL1-5/Naa10) or Naa10p-R82A (CL1-5/R82A) grew small tumors (Figure S3A). The ability of these tumors to retain the expression of Naa10p or Naa10p-R82A was examined (Figure S3B). No metastases were macroscopically visible in the lungs of these mice; longer monitoring times would be needed to observe such secondary tumors. However, the mice had to be sacrificed after 4 weeks due to large subcutaneous tumors according to the guidelines of the Animal Ethic Committee. To circumvent this problem, we isolated cells from the lung tissue at the endpoint of the study and cultured them in the presence of G418 for 2 weeks to select for transfected cells. The control cells formed 1–60 colonies per lung compared with 0–7 in the CL1-5/Naa10 group and 0–4 in the CL1-5/R82A group (Figure S3C). PCR analysis of human-specific *Alu* element further supported these findings, in that the Naa10p and Naa10p-R82A groups had relatively few human cancer cells in the lungs compared with the control group (Figure S3D).

To further evaluate the role of Naa10p in metastasis, we used murine breast cancer cell line 4T1, which shows frequent lung metastasis after orthotopic fat pad inoculation. In order to avoid the effects of different sizes of primary tumors, we surgically removed fat pad tumors after the tumor size exceed 1 cm^3. Mice were sacrificed after 2 weeks and lung metastases were examined by macroscopic observation and luciferase activity. Naa10p or Naa10p-R82A expression significantly diminished lung metastases (Figures 3A and 3B). Furthermore, axillary lymph node enlargements were found to be decreased in the Naa10p and Naa10p-R82A groups (Figure 3C). We also

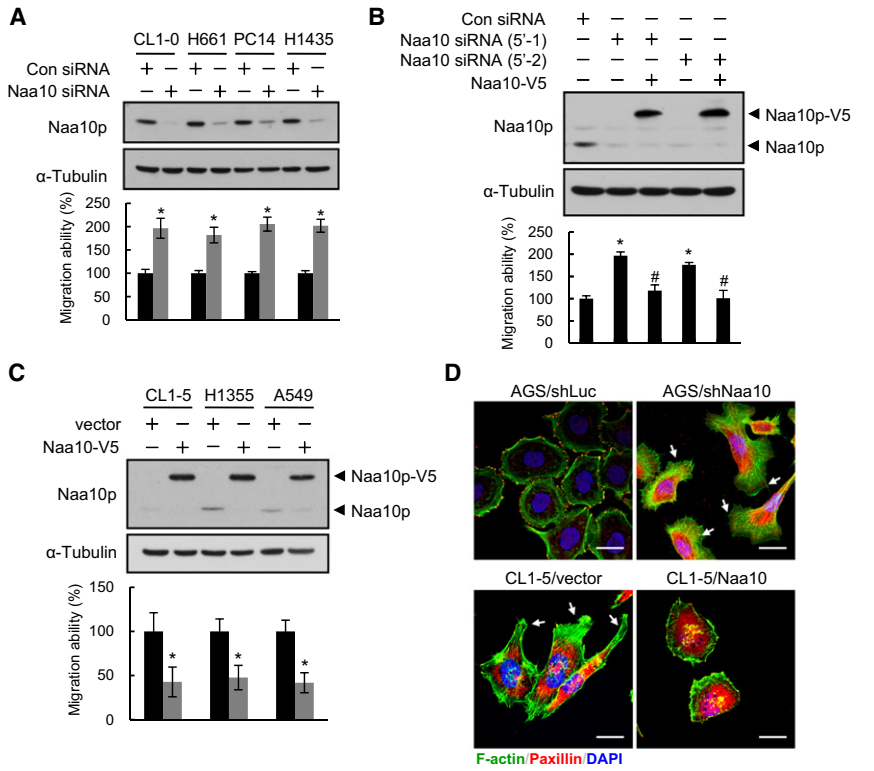

Figure 2. Naa10p Suppresses Mobility of Cancer Cells

(A) Transwell migration assay of lung cancer cell lines including CL1-0, H661, PC14, and H1435 transfected with either a pool of four control siRNA or Naa10p siRNA. The fold difference represents the mean of triplicate experiments compared with control siRNA-treated cells. *$p < 0.05$.

(B) Transwell migration assay of CL1-0 cells transfected with two different Naa10 siRNAs targeting 5′-UTR of Naa10 mRNA with or without cotransfected with Naa10-V5 as indicated. The fold difference represents the mean of triplicate experiments compared with control siRNA-treated cells. *$p < 0.05$.

(C) Transwell migration assays of lung cancer cell lines including CL1-5, H1355, and A549 either transfected with control vector or Naa10p-V5. The fold difference represents the mean of triplicate experiments compared with control cells. *$p < 0.05$.

(D) The cellular morphology is altered in cells with overexpressed or downregulated Naa10p expression. CL1-5 and AGS cells were transfected with either Naa10p-V5, Naa10p shRNA, or their respective controls, as indicated. Cells were fixed and stained for F-actin (green) and paxillin (red). Nuclei were counterstained with DAPI (blue). Scale bars, 20 μm.

(E) A549 cells were transfected with NATH-Myc, Naa10p-V5, and R82A-V5 either alone or in combination with each other, as indicated and subjected to transwell migration assay. The fold difference represents the mean of triplicate experiments compared with control cells. *$p < 0.05$.

(F) CL1-5 cells were transfected with Naa10-V5 or Naa11-V5, and the transwell migration assay was performed. Each data point is representative as mean of triplicate experiments. *$p < 0.05$.

(G) CL1-0 cells were infected with lentivirus carrying shRNA for either Naa10 or Naa11 and subjected to transwell migration assay. RT-PCR was performed using Naa10- or Naa11-specific primers. The fold difference represents the mean of triplicate experiments compared with control cells. *$p < 0.05$. Error bars indicate SD.

See also Figure S2.

examined the presence of 4T1 cells in the peripheral blood and found significantly fewer cell numbers in the Naa10p and Naa10p-R82A groups (Figure 3D). We also examined the expression of Naa10p or Naa10p-R82A in colonies derived from either group to see if these colonies retained the expression of Naa10p or Naa10p-R82A. In all of the colonies, only four from the ten member Naa10p group and one from the nine member Naa10p-R82A group still expressed Naa10p/Naa10p-R82A (Figure S3E). In order to examine lung colonization, lung adenocarcinoma cell line A549 was injected into the lateral tail vein of NOD-SCID mice. Six weeks postinjection, significantly lower metastatic nodules were formed in the A549/Naa10 group compared with the A549/vector group (Figure S3F). Our data indicate that Naa10p expression suppresses tumor cell growth, extravasation, intravasation, and lung colonization.

Naa10p Alters the Activation Status of Cdc42 and Rac1

Rho family GTPases such as Rac1, Cdc42, and RhoA control actin dynamics and play key roles in cell migration. To determine whether the function of Naa10p in cell migration is mediated through the Rho family of GTPases, we compared the levels of GTP-bound RhoA, Rac1, and Cdc42 in the CL1-0 and CL1-5 cell lines. CL1-5 cells transfected with Naa10p or Naa10p-R82A resulted in lower levels of GTP-bound Rac1 and Cdc42 when compared with controls, while CL1-0 cells transfected with a siRNA pool directed against Naa10p resulted in higher levels of GTP-bound Rac1 and Cdc42 (Figure 4A). This result was further confirmed by using individual siRNA from the pool to knock down Naa10p and observed similar elevation of GTP-bound Rac1 and Cdc42 (Figure S4A). To exclude the possibility of off-target effects, we reintroduced Naa10p in to the cells, bringing the GTP-bound Rac1 and Cdc42 level back to normal

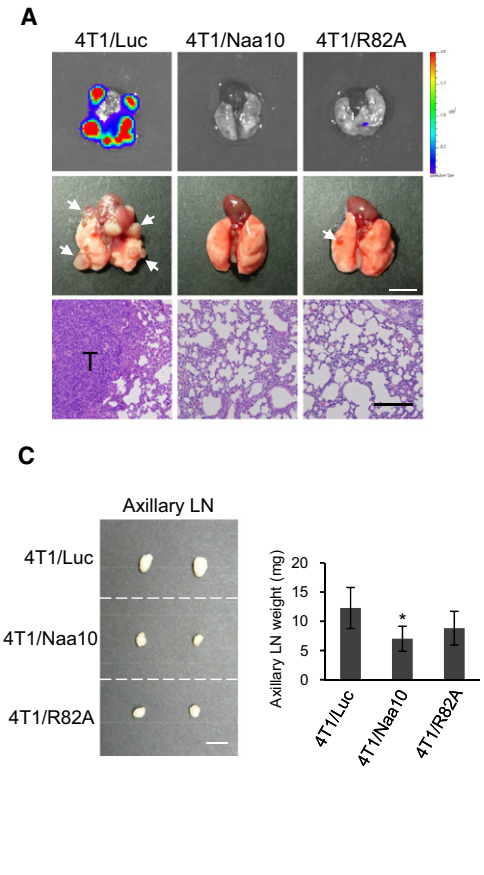

Figure 3. Naa10p Suppresses Metastatic Colonization in Animal Models

(A) Lungs were isolated and examined after spontaneous metastasis assay. Upper panel: representative luciferase activity image. Middle panel: photos of lungs. Lower panel: H & E staining of lung metastatic tumors. Scale bar, middle panel: 5 mm, lower panel: 100 μm.

(B) Upper panel: number of surface metastases in lungs of mice. Lower panel: quantification of luciferase activity. Photon counting analysis of lung nodules was performed after inoculation with luciferin. *p < 0.05; n = 7 mice per group.

(C) Macroscopic analysis of axillary lymph nodes. Lymph node was photographed and weighted after removal. Error bars indicate SD. Scale bar, 0.5 cm. *p < 0.05.

(D) Naa10p reduced intravasation of 4T1 cells. The intravasation of tumor cells was determined from the blood culture of mice. Number of colonies was counted after grown in G418-containing media for 2 weeks.

See also Figure S3.

(Figure S4B). The levels of GTP-bound RhoA, however, remained unchanged. To elucidate the importance of decreased GTP-bound Rac1 and Cdc42 in Naa10p-regulated cell mobility, we expressed constitutively active Rac1 ($Rac1^{V12}$) and Cdc42 ($Cdc42^{V12}$) in CL1-5 cells with stable Naa10p expression. Both $Rac1^{V12}$ and $Cdc42^{V12}$ were found to be able to reverse the effect of Naa10p to bring the migration ability back to normal levels (Figure 4B). Furthermore, dominant negative Rac1 ($Rac1^{N17}$) and Cdc42 ($Cdc42^{N17}$) suppressed the migration ability induced by shRNA-mediated Naa10p knockdown in MCF-7 cells (Figure 4C). These data suggest that Naa10p regulation of cell migration depends on its ability to modulate Rac1 and Cdc42 activity.

Naa10p Interacts with βPIX

Our results suggest that Naa10p regulates cell mobility in an acetyltransferase-independent manner, possibly through the activation of Rac1 and Cdc42. However, we did not detect any direct association between Naa10p and Rac1 or Cdc42 (data not shown), which indicates that other binding partners such as GEFs or GAPs are required for Naa10p's regulation of Cdc42 and Rac1 activity.

To investigate the Naa10p-dependent regulation of Rac1 and Cdc42, we performed coimmunoprecipitation with V5-tagged Naa10p and known regulators of Rac1 and Cdc42 activity. We found that the PIX proteins (αPIX and βPIX) were able to bind to Naa10p. The PIX proteins are a group of GEFs known to regulate both Rac1 and Cdc42. In contrast, TIAM1, another GEF specific for Rac1/Cdc42, did not associate with Naa10p (Figure 5A). Previous studies have shown that αPIX is expressed primarily in hematopoietic cells and muscle, whereas βPIX is ubiquitously expressed (Koh et al., 2001; Manser et al., 1998). In agreement with these findings, we detected the interaction between endogenous Naa10p and βPIX (Figure 5B) by coimmunoprecipitation. In addition, in vitro pull-down assays using recombinant GST-Naa10p (generated from *Escherichia coli*) and in vitro-transcribed/translated βPIX further suggested a direct association between Naa10p and βPIX (Figure 5C). A similar association occurred between Naa10p and αPIX (Figure 5C). Moreover, Naa11p and Naa15p failed to coimmunoprecipitate with αPIX or βPIX, implying a highly specific interaction between Naa10p and α/βPIX (Figures S5A and S5B).

The Naa10p protein contains a conserved acetyltransferase domain. It was also reported that residues 1–58 in the N terminus were responsible for the Naa15p interaction, and that the last 50 residues in the C terminus mediated interaction with β-amyloid precursor protein (Asaumi et al., 2005). To characterize the mechanism by which Naa10p and αPIX/βPIX interact, a series of expression constructs were generated for the wild-type full-length and truncated mutants of Naa10p (Figure 5D). The αPIX and βPIX could be pulled down by full-length Naa10p, N-terminal deleted Naa10p (residues 59–235), and the Naa10p-acetyltransferase domain (residues 60–130), but not by C-terminal Naa10p (residues 131–235) (Figure 5E; Figure S5C). These results indicate that the acetyltransferase domain is the primary site for αPIX and βPIX interaction.

To map the Naa10p binding region of βPIX, we used a series of βPIX truncation mutants (Figure 5D). Coimmunoprecipitation results revealed that all constructs with a GIT-binding domain (GBD) interacted with Naa10p (Figure 5F). This domain is

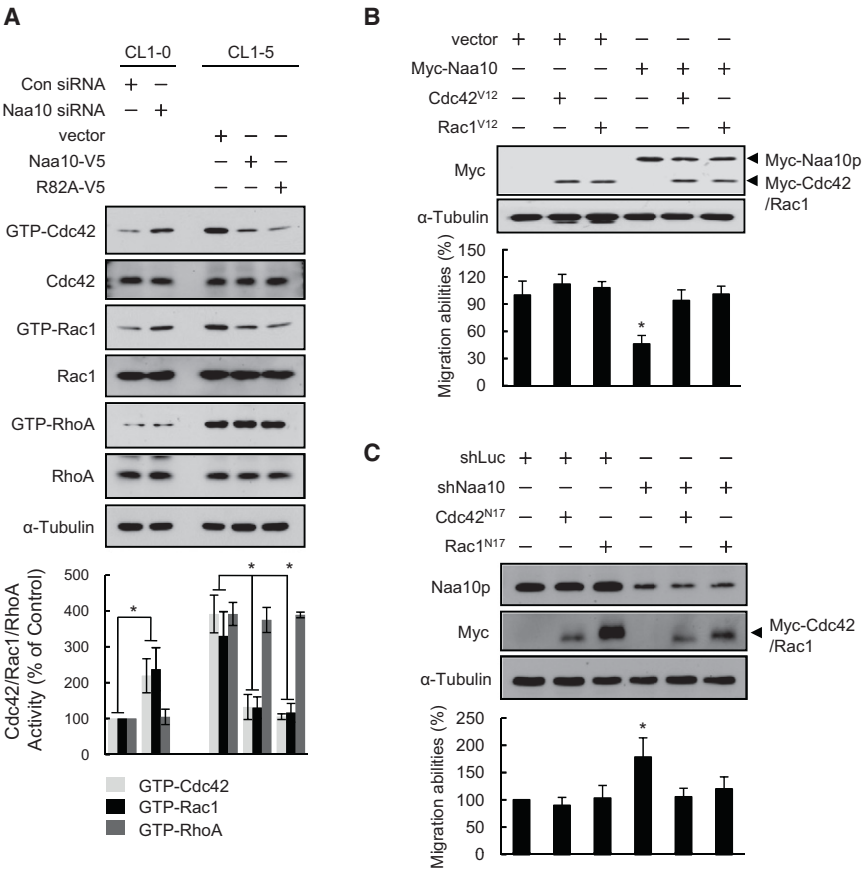

Figure 4. Naa10p Alters the Activation Status of Cdc42/Rac1

(A) CL1-5 and CL1-0 cells were transfected with Naa10-V5, R82A-V5, Naa10 siRNA, or their corresponding controls as indicated. GTP-Cdc42 and Rac1 were assayed using GST-PBD. GTP-RhoA was assayed using GST-TBD. Each experiment was repeated three times and the amounts of GTP-Cdc42/Rac1/RhoA were analyzed by densitometer. Upper panel: representative blot of the Cdc42/Rac1/RhoA activity assay. Lower panel: quantification and statistical analysis of the GTP-Cdc42/Rac1/RhoA. *$p < 0.05$.

(B) Cell migration of CL1-5 cells transiently transfected with either Naa10-V5 or control vector, or together with Myc-tagged $Cdc42^{V12}$ or $Rac1^{V12}$. The fold difference represents the mean of triplicate experiments compared with control cells. *$p < 0.05$.

(C) Cell migration of MCF-7 cells infected by lentivirus carrying either Naa10 shRNA or control luciferase shRNA, either alone or after transfection with Myc-tagged $Cdc42^{N17}$ or $Rac1^{N17}$. The fold difference represents the mean of triplicate experiments compared with luciferase shRNA infected cells. *$p < 0.05$. Error bars indicate SD.

See also Figure S4.

conserved between αPIX and βPIX, and as expected, we found that Naa10p and αPIX interacted in the same fashion (Figure S5D). Taken together, these results show that the Naa10p acetyltransferase domain interacts with the αPIX/βPIX GIT-binding domain. We next examined whether βPIX was acetylated by Naa10p. Since their interaction was independent of Naa15p, βPIX was less likely to be *N*-α-acetylated by Naa10p. Naa10p has also been reported to function as an *N*-ε-acetyltransferase (Lim et al., 2006, 2008). Thus, we examined whether Naa10p mediated *N*-ε-acetylation of βPIX. βPIX were immunoprecipitated from cells with Naa10p overexpression or knockdown by siRNA, and the acetylated βPIX was detected by antibodies against acetyl lysine. However, we could not detect any acetylated βPIX even with three different antibodies (Figure S5E), indicating that Naa10p did not *N*-ε-acetylated βPIX.

Naa10p Disrupts the Interaction with GIT to Inhibit Rac1/Cdc42 Activation and Cell Migration

Our results indicate that Naa10p regulates cellular migration through the interaction with PIX. To further explore this possibility, we transfected Naa10p either alone or in combination with βPIX in 293T cells. As expected, single transfection of βPIX resulted in increased Cdc42 and Rac1 activity. Interestingly dual transfection of Naa10p and βPIX only caused a modest increase of the activity compare to the untransfected control (Figure 6A).

We also conducted single or dual transfection (as noted in Figure 6B) in the highly migratory cell line CL1-5. Interestingly, individual transfection of GIT1, αPIX, or βPIX was sufficient to increase cell migration, while single transfection of Naa10p resulted in a significant decrease of cell migration. In consistence with the above findings, dual transfection of Naa10p in combination with GIT1, αPIX or βPIX resulted in similar migration compared with the untransfected control (Figure 6B). Hence overexpression of Naa10p is sufficient to prevent PIX dependent activation of Cdc/Rac activity and cell migration.

Similary, knockdown of βPIX alone was sufficient to suppress Rac1 and Cdc42 activity and inhibited cell migration and the metastatic ability of CL1-5 cells (Figures S6A, S7A, and S7D–S7G). Furthermore, overexpression of αPIX or βPIX increased the migration ability of CL1-0 cells (Figure S7B). Consistently, shRNA specific for αPIX or βPIX decreased the migration ability of Naa10p knockdown AGS cells (Figure 6C). These data suggested that αPIX and/or βPIX are important for Naa10p to regulate small G protein activity and cell migration.

Previous studies have shown that GIT proteins are involved in guiding PIX proteins to the cell periphery or focal adhesions where they activate Rac1 or Cdc42 (Koh et al., 2001). In order to evaluate the importance of GIT proteins in our cell model, we used shRNAs to knock down GIT1 and GIT2 separately and estimated the levels of GTP-bound Rac1/Cdc42. Both GIT1 and GIT2 were expressed in CL1-5 cells and affected the GTP-bound level of Rac1/Cdc42 and cell migration when diminished by shRNAs. Furthermore, simultaneous knockdown of both GITs further suppresses the activity of Rac1/Cdc42 (Figure S6B) and cell migration to a more significant level

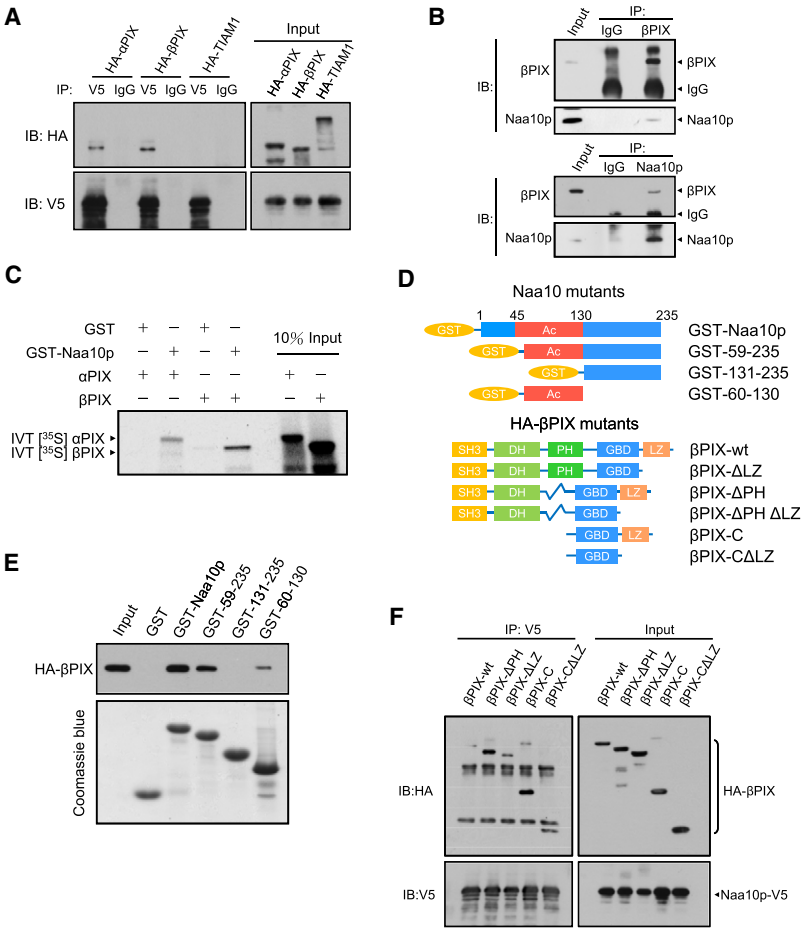

Figure 5. Naa10p Associates with αPIX and βPIX

(A) 293T cells were transfected with Naa10-V5 together with HA-αPIX, HA-βPIX, or HA-TIAM1 as indicated. Cell lysates were immunoprecipitated with anti-V5 or normal mouse IgG antibody, respectively.

(B) Endogenous interaction between βPIX and Naa10p. AGS cell lysates were immunoprecipitated with immobilized anti-βPIX or anti-Naa10p protein A beads and immunoblotted with anti-βPIX and anti-Naa10p antibody.

(C) Purified recombinant GST-Naa10p was incubated with [^{35}S]-αPIX or [^{35}S]-βPIX in the presence of glutathione-Sepharose. The washed precipitates were subjected to SDS-PAGE, and [^{35}S]- αPIX or [^{35}S]-βPIX were detected by autoradiography.

(D) Schematic diagram represents expressed fragments of Naa10p and βPIX. Naa10p containing acetyltransferase domain (Ac) and βPIX containing SH3, Dbl (DH), and Pleckstrin (PH) homology domains, a Cat (Cool-associated tyrosine phosphosubstrate)/Git (G protein-coupled receptor kinase interactor)-binding (GBD) domain and a leucine zipper (LZ).

(E) HA-βPIX expressed in 293T cells was incubated with GST-fused Naa10p fragments in the presence of glutathione-Sepharose. The washed precipitates were subjected to SDS-PAGE, followed by immunoblotting with anti-HA antibody. Protein loading and purities were confirmed by Coomassie blue staining.

(F) HA-tagged fragments of βPIX as indicated were coexpressed with Naa10-V5 in 293T cells and immunoprecipitated with anti-V5 antibody. The presence of associated βPIX protein was assessed by immunoblotting using anti-HA antibody.

(A–F) In each experiment, 10% of each lysate was used as input. See also Figure S5.

(Figure S7C, $p < 0.005$). These results suggest that both GIT1 and GIT2 promote Rac1/Cdc42 activation and cell migration in CL1-5 cells. Since Naa10p binds to the GIT-binding domain of PIX proteins, we evaluated whether Naa10p could disrupt the association between αPIX or βPIX and GIT proteins. Our results indicated that the ability of GST-βPIX to associate with Flag-tagged GIT1 and GIT2 was decreased in the presence of V5-tagged Naa10p (Figure 6D) or Naa10p-R82A (Figure S6C). However, the ability of GST-βPIX to associate with HA-tagged PAK1 or PAK3, which interacts with the SH3 domain of the PIX proteins, was not changed in the presence of Naa10p or Naa10p-R82A (Figure S6D). Moreover, GST-Naa10p lost its ability to associate with Myc-tagged αPIX in the presence of Flag-tagged GIT1 (Figure S6E). Consistent with these findings, the amount of βPIX-associated GIT1 was higher in low Naa10p-expressing CL1-5 cells compared with the high Naa10p-expressing CL1-0 cells (Figure 6E). This competition could also be observed in the cell migration ability, where overexpression of Flag-tagged GIT1, but not the Myc-tagged PAK1, increased the migration ability of Naa10p-overexpressing CL1-5 cells (Figure 6B). Similarly, shRNA specific for GIT1 reduced the migration ability of Naa10p knockdown AGS cells (Figure 6C). To determine whether Naa10p could influence the localization of αPIX and βPIX, we evaluated the distribution of endogenous βPIX by immunofluorescence staining analysis. βPIX was detected in the puncta of membrane protrusions in A549 cells, but was dramatically decreased in cells with Naa10p-V5 expression (Figure 6F). Furthermore, transfection of shRNAs against αPIX or βPIX in Naa10p-knockdown AGS cells resulted in a less protrusive phenotype. Ectopic overexpression of αPIX or βPIX in CL1-5/Naa10 cells resulted in a more protrusive phenotype, similar to that observed in the CL1-5 control cells (Figure S6G). We also noted a slight decrease of βPIX in the membrane fraction of CL1-5/Naa10 cells (Figure 6G). Similarly, a substantial portion of βPIX was detected in the membrane fraction of CL1-0 cells when Naa10p was depleted (Figure S6F). These data suggest that Naa10p competes against GIT for binding to PIX, preventing the translocation of PIX to the plasma membrane. This results in decreased Rac1/Cdc42 activation and decreased cell mobility.

To elucidate the role of Naa10p and PIX proteins in metastasis, we generated GFP- and luciferase-expressing CL1-5 cells (CL1-5GL) and transfected them with an empty vector, Naa10p-V5 alone, or in combination with αPIX or βPIX (Figure 7A). Cells were injected into the tail vein of NOD-SCID mice and the mice were monitored for the formation of lung tumors. Four weeks after injection, the number of lung metastatic nodules was determined by bioluminescence activity. Large numbers of metastatic nodules were observed in animals injected with control CL1-5GL cells, whereas few nodules were

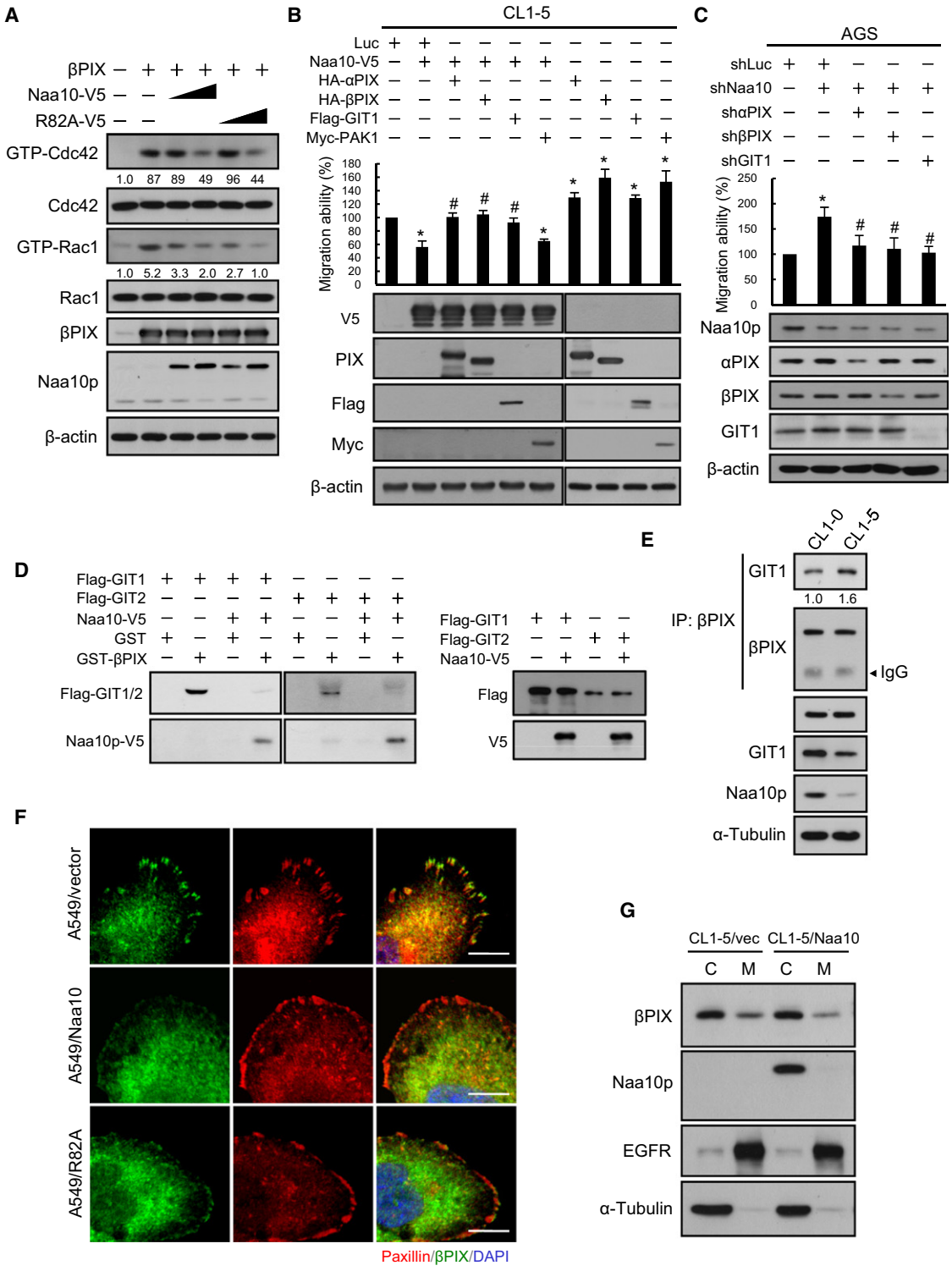

Figure 6. Naa10p Disrupts the Interaction between PIX and GIT to Inhibit Cdc42/Rac1 Activation and Cell Migration

(A) 293T cells were transfected with either control vector or HA-βPIX alone or together with different amount of Naa10-V5 or R82A-V5. Cell lysates were harvested and subjected to GST-PBD pull-down assay. The amounts of GTP-Cdc42/Rac1 were analyzed by densitometer.

(B) CL1-5 cells were transfected with control vector, Naa10-V5, HA-αPIX, HA-βPIX, Flag-GIT1, and Myc-PAK1 as indicated and subjected to transwell migration assay. The fold difference represents the mean of triplicate experiments compared with control cells. *$p < 0.05$ as compared with control cells. #$p < 0.05$ as compared with Naa10-V5 transfected CL1-5 cells.

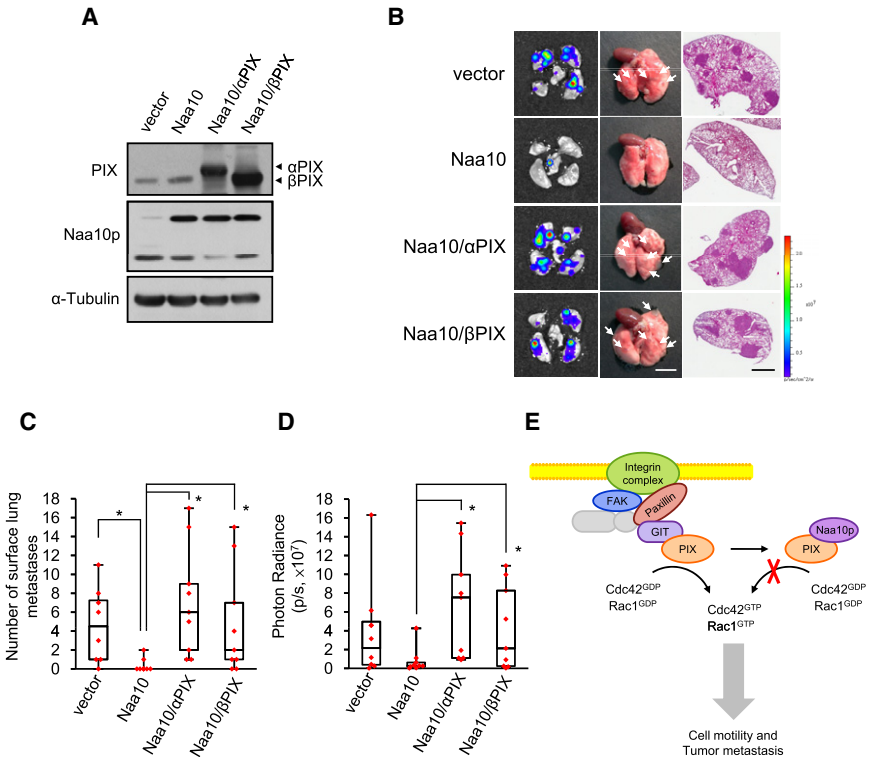

Figure 7. Expression of PIX Proteins Rescued Cancer Cell Metastatic Ability Suppressed by Naa10p

(A) Establishment of CL1-5/luciferase cells stably expressing Naa10-V5 and Naa10-V5 plus αPIX or βPIX. Expression of Naa10p was detected using a chicken anti-Naa10p polyclonal antibody (ab51787, Abcam). αPIX and βPIX were detected using a rabbit anti-PIX polyclonal antibody (AB3829, Chemicon).

(B) Representative photographs of lungs taken 28 days after tail vein injection of the cells into NOD-SCID mice. Left panel: Photon counting analysis of lung nodules was performed after inoculation with luciferin. Middle and right panel: Representative lungs and H & E staining of lung metastatic tumors in each group are shown. Scale bar, middle panel: 5 mm, right panel: 2 mm.

(C) Number of surface metastases in lungs of mice (n = 8–9 per group) 4 weeks after tail vein injection of cells. *p < 0.05.

(D) Quantitative photon counting analysis of lung metastases in each group. *p < 0.05.

(E) Schematic representation depicting the effects of Naa10p on PIX localization and Cdc42/Rac1 activation. In migrating cells, PIX colocalizes with GIT/Paxillin at focal adhesions. This allows PIX to activate Cdc42/Rac1. Active Cdc42/Rac1 induces actin dynamic change, contributing to cell migration. Naa10p competes with GIT binding to PIX and sequesters PIX from focal adhesion, blocking Cdc42/Rac1 activation and cell migration. See also Figure S7.

detectable in animals injected with Naa10p-V5-expressing cells. As predicted, overexpression of αPIX or βPIX increased the number of metastatic nodules in ARD-V5-expressing CL1-5GL cells (Figure 7B). Quantitative data (the number of surface lung metastases and level of photon radiance), shown in Figures 7C and 7D, confirmed these findings. Together this indicates that the Naa10p-mediated regulation of PIX proteins is an important event in the formation of lung metastasis.

DISCUSSION

Tumor metastasis is the main cause of cancer-related death. Identifying genes regulating cell migration and metastasis is crucial to the understanding of this process. In recent years, the Rac1 and Cdc42 pathways have been extensively studied in the context of metastasis. The complex interplay between its regulators and effectors results in the establishment of membrane protrusions and eventual cell migration. The PIX family of proteins has an important function in this process, acting as a GEF and scaffold protein between Rac1/Cdc42 and their effectors. We have identified Naa10p as a regulator of PIX and suppressor of tumor cell migration and/or metastasis. We found that Naa10p and GIT bind to the same site of the PIX protein. In the presence of Naa10p, GIT proteins fail to bind to the PIX proteins, disrupting the translocation of PIX to the plasma membrane (Figure 7E). Therefore, high expression of Naa10p restricts cell mobility and decreases the possibility of cancer cell metastasis in vivo.

Recently, Lee et al. have used Naa10p antibody to analyze a small cohort of lung adenocarcinoma and observed

(C) Cell migration of AGS cells infected with lentivirus carrying shRNA for either Naa10p or luciferase, or together with shRNA for αPIX, βPIX, or GIT1. The percentage of migrated cells compared with cells receiving shRNA for luciferase is shown. The knockdown efficiency was shown by western blot analysis using specific antibodies. *p < 0.05.

(D) 293T cell lysates containing Flag-GIT1, Flag-GIT2, or Naa10-V5 were incubated with either GST or GST-βPIX as indicated. Left panel: Pull-down products were washed and subjected to SDS-PAGE. GIT1, GIT2, and Naa10p were detected by immunoblotting using specific antibodies. Right panel: 10% loading input for each group.

(E) βPIX-associated GIT1 were examined in CL1-0 and CL1-5 cells. Cell lysates were immunoprecipitated with anti-βPIX and immunoblotted with anti-GIT1 antibody.

(F) Representative photographs for immunofluorescence staining of endogenous βPIX in A549 cells stably expressing Naa10-V5 or control vector. Arrows indicate βPIX-enriched cell protrusions. Nuclei were counterstained with DAPI. Scale bar, 10 μm.

(G) Subcellular fractionation was performed with the CNM compartmental protein extraction kit (K3012010, BioChain Institute, Inc., Hayward, CA). The subcellular distribution of Naa10p and βPIX was determined by immunoblotting. EGFR was used as the membrane marker, and α-Tubulin was used as the cytosolic marker. M, membrane fraction; C, cytosolic fraction. Error bars indicate SD. See also Figure S6.

a correlation between Naa10p expression and patient survival (Lee et al., 2010). However, since Naa10p is known to have several variants and homolog (Bilton et al., 2006), it is possible that Lee's antibody raised against full-length Naa10p may also recognize these Naa10p family proteins due to their highly conserved acetyltransferse motif. Here, we used a specific antibody which recognizes the C-terminal region of Naa10p (aa 155–235) to analyze 318 lung tumor specimens and found a reverse correlation with patient overall survival ($p = 0.0044$) and disease-free survival ($p = 0.0039$). We have also observed a significant inverse correlation of Naa10p expression and patient survival in female adenocarcinoma population. However, Naa10p expressions are not significantly correlates with patient survival of male or SCC patients (Figures S1C and S1D). The inconsistent results may be due to the antibody specificity and/or the different compositions of patient cohorts. Collectively, our results based on specific antibody and multiple lines of clinical evidence support that Naa10p is a suppressor of tumor progression.

N-α-acetylation is one of the most common post-translational modifications in eukaryotes. The major *N*-α-acetyltransferase in human cells is composed of Naa10p and Naa15p (Polevoda and Sherman, 2000). There have also been reports indicating that Naa10p can function as an *N*-ε-acetyltransferase (Lim et al., 2006, 2008). However, it remains questionable as to whether one enzyme could catalyze both *N*-α- and *N*-ε-acetylation. Here, we report that Naa10p can exert its function independent of its enzymatic activity. We found that Naa10p suppressed cell migration in the absence of Naa15p. Unlike Naa10p depletion by siRNAs or shRNA, Naa15p depletion by shRNA specific for Naa15p did not affect cell migration (data not shown). Furthermore, a Naa10p mutant without acetyl-CoA binding ability retained its migration inhibiting capability. We propose that Naa10p regulates cell migration by interacting with the PIX family of proteins. Although the interaction with PIX occurs with the acetyltransferase domain of Naa10p, we did not observe any detectable level of acetylated βPIX in cells under the influence of Naa10p-V5 or in the presence of specific shRNA against it, indicating that βPIX may not be an *N*-ε-acetylation target of Naa10p.

Our data suggest that Naa10p regulates cell migration through the Rac1/Cdc42 pathway. PIX is a well-studied regulator as a GEF of both Rac1 and Cdc42. Our current hypothesis is that Naa10p binds and regulates the cellular localization of PIX thereby preventing the activation of Rac1/Cdc42. In addition to acting as a GEF, PIX can function as scaffold protein within the focal adhesion complex. Previous studies have shown that PAK, the major component of the Rac1/Cdc42 pathway can bind to the SH3 domain of PIX (Manser et al., 1998). Hence, PIX does not only activate PAK, it plays a central role in bringing PAK to the focal adhesion complex. Interestingly, PIX has been shown to activate PAK independently of Rac1/Cdc42 activity, possibly through the interaction of the other members of the focal adhesion complex. In addition to PAK, PIX can associate with GIT, another scaffold protein of the focal adhesion complex. GIT function as the link between paxillin and the PIX complex (Zhao et al., 2000). The GIT family consists of two highly similar proteins: GIT1 and GIT2 (Premont et al., 1998, 2000). Both bind directly to paxillin, and thereby localized to focal adhesions. Our result indicates that both GIT proteins may be involved in Rac1/Cdc42 activation and cell migration potentials in a similar manner, as the depletions of both GIT1 and GIT2 suppress GTPase activation and reduces cell migration more significantly than depletion of either one alone (Figure S6B). The underlying mechanism is speculated to be that GIT1 and GIT2 could substitute for each other in directing PIX proteins to focal adhesions through interaction with paxillin. Upon depletion of one GIT proteins, the other one is still capable of directing localization of PIX proteins. We propose that Naa10p competes with GIT for the binding of PIX. Since this interaction is required to bring PIX to the focal adhesion, overexpression of Naa10p reduces the amount of PIX at the cellular membrane (Figures 6F and 6G), prevents the activation of Cdc42/Rac (Figure 4A) and decrease cell migration.

Although our data suggest GBD domain is the primary site for Naa10p interaction, the other domains or other PIX-associated protein may also affect the interaction between Naa10p and PIX. Given that PH domain-deleted βPIX showed a reduced affinity to Naa10p (Figure 5F), it is possible that proteins associated with PH domain may help providing proper structure for Naa10p docking at the GBD domain. Similar situations may occur at other domains, since SH3 and LZ domain deletion also seems to affect the interaction between αPIX and Naa10p (Figure S5D). However, our results suggest that Naa10p binding only affects the interaction between GIT1/2 and PIX but not PAK1/3, which interacts with PIX at the SH3 domain. This observation supports that the GBD domain was the major site, even not the only site, for the Naa10p interaction.

The role of Naa10p in cell migration is likely to be complex. Although our study indicates an acetylation-independent mechanism of Naa10p in regulating cell mobility, we cannot rule out the acetylation-dependent mechanism. As shown in Figure S2F, the Naa10p-R82A mutant may still have some remaining function to acetylate some other specific proteins that participate in cell mobility control. Recently, myosin light chain kinase (MLCK) was found to be another Naa10p target (Shin et al., 2009). Naa10p acetylated MLCK and suppressed its activity, which resulted in decreased cell mobility. Interestingly, myosin light chain is one of the targets of the PAKs (Chew et al., 1998; Coniglio et al., 2008), which suggests a possible involvement of the PIX-GIT-Paxillin complex in this process. PAKs could either be directly activated by Rac1 and Cdc42 binding (Knaus et al., 1998) or induced by GIT-PIX-PAK complex formation (Loo et al., 2004). Our data suggest that both of these processes can be suppressed by Naa10p. A study by Shin et al. (2009) and our findings emphasize the importance of Naa10p as an important regulator of cell migration and metastasis.

Naa10p was found to express in tumor tissues. However, the role of Naa10p in tumorigenesis is controversy. Although growth-promoting activity associated with Naa10p function is well in line with published evidence, there is also report suggested that different Naa10 siRNAs used may result in complete opposite effect (Yi et al., 2007). Lee et al. reported that Naa10p promotes tumorigenesis in lung cancer cells, and suggest that Naa10p is helping the DNMT to silence tumor suppressors. However, Naa10p maybe act through different mechanism in normal epithelium and tumor cells. Recently, Naa10p has been shown to suppress tumorigenesis through acteylating TSC2 and induce autophagy of tumor cell (Kuo et al., 2010). Kuo

et al. and our in vivo results had also shown significant smaller tumor size of xenografts in Naa10p overexpression cells (Figure S3A). In line with our clinical results (Table 1), Kuo et al. had also observed a significant lower Naa10p expression in breast cancer specimens and lymph node metastases. Although the exact roles of Naa10p in tumorigenesis and/or tumor progression remain to be elucidated, we showed here that Naa10p act as a metastasis suppressor during tumor progression.

In conclusion, we have shown that Naa10p is a good prognostic factor whose overexpression is associated with increased survival and decreased lymph node metastasis. We propose that Naa10p can act as a tumor migration and/or metastasis suppressor by binding to the GIT binding domain of PIX. Our data indicate that Naa10p has the opposite effect of GIT to prevent membrane protrusion, cell migration, and lymph node metastasis.

EXPERIMENTAL PROCEDURES

Immunohistochemistry

Tissues utilized were obtained with informed consent and the study was conducted under the approval of the Institutional Review Boards of National Taiwan University Hospital and Kaohsiung Medical University Hospital. Tissue was fixed in formalin and subsequently was dehydrated, paraffin embedded, and sectioned. Tissue sections were immunostained as described previously (Su et al., 2004). A four-point staining intensity scoring system was devised for determining the relative expression of Naa10p in cancer specimens; the staining intensity score ranged from 0 (no expression) to 3 (maximal expression). The results were classified into two groups according to both the intensity and extent of staining: in the low-expression group, either no staining was present (staining intensity score = 0) or positive staining was detected in less than 10% of the cells (staining intensity score = 1), and in the high-expression group, positive immunostaining was present in 10%–25% (staining intensity score = 2) or more than 25% of the cells (staining intensity score = 3). All of the immunostaining results were reviewed and scored independently by two pathologists. The histological diagnosis of lung adenocarcinoma was made according to the recommendations of the World Health Organization (Gibbs and Thunnissen, 2001). Tumor size, local invasion, lymph node metastasis, and final disease stage were determined as described previously (Sobin and Fleming, 1997). Follow-up of patients was carried out up to 200 months.

siRNA Transfection

On-Target plus siRNAs for human Naa10p, as well as Risc-free control siRNA, were from Dharmacon Research (Lafayette, CO). Cells were transfected with siRNAs as indicated for 18–24 hr using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) followed by a change of media. Two to three days after siRNA transfection, cells were subjected to migration assays. Reduced expression by siRNA was monitored by immunoblotting with Naa10p antibody.

Two-Chamber Migration Assay

Cell mobility was determined using the modified two-chamber migration assay (8 μm pore size, BD Biosciences, Bedford, MA) according to the manufacturer's instructions. About 2×10^4 cells were seeded into the upper chamber and allowed to migrate into the lower chamber for 18–24 hr. Cells in the upper chamber were carefully removed using cotton buds and cells at the bottom of the membrane were fixed and stained with crystal violet 0.2%/methanol 20%. Quantification was performed by counting the stained cells.

Immunofluorescence Microscopy

Cells were grown on coverslips and fixed in 4% paraformaldehyde, permeabilized, and stained with primary antibodies followed by secondary FITC or Alexa 555-conjugated mouse or rabbit antibodies. F-actin was detected with FITC-phalloidin (Invitrogen). Slides were examined and photographed using a Zeiss Axiophot fluorescence microscope. Nuclei were counterstained with 4′,6-diamino-2-phenylindole (DAPI).

Immunoprecipitation

Cells were lysed by brief sonication (3 × 1 s pulses) in coimmunoprecipitation buffer (20 mM Tris [pH 8.0], 150 mM NaCl, 1 mM EDTA, 0.5% NP-40 supplemented with protease [Complete, Roche] inhibitors). Lysates were centrifuged for 20 min at 10,000 × *g* and the resulting supernatant was precleared by incubation with immobilized Protein A/G gel (25 μl; Pierce, Rockford, IL) for 1 hr at 4°C. The precleared supernatant was subjected to overnight immunoprecipitation using the indicated antibodies or control IgG antibodies at 4°C. The next day, protein complexes were collected by incubation with 25 μl of immobilized Protein A/G gel for 1 hr at 4°C. The collected protein complexes were washed four times with coimmunoprecipitation buffer and eluted by boiling in protein sample buffer under reducing conditions, after which proteins were resolved on SDS–PAGE and analyzed by western blot.

Spontaneous Metastasis Assay

Seven female BALB/c mice were fat pad injected with 4T1/Luc cells overexpressing Naa10p, Naa10p-R82A, or control vector (1×10^5 cells with Matrigel at 5 mg ml^{-1} of PBS in a volume of 200 μl). Tumor growth was monitored twice a week by Vernier caliper measurement of two perpendicular tumor diameters (L and W). Tumor volume was calculated using the formula $LW^2/2$. After the tumor volume exceeded 1 cm^3, the primary tumors were surgically removed. Lungs were isolated 2 weeks after the surgery and examined. The tumor colonies were counted 3 weeks later. All animal work was performed in accordance with protocols approved by the Institutional Animal Care and Use Committee of the College of Medicine, National Taiwan University.

Statistical Analysis

Results are expressed as the mean ± SD. We utilized a two-tailed, unpaired Student's t test for all pair-wise comparisons. Survival curves were obtained using the Kaplan-Meier method.

p values less than 0.05 were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at doi:10.1016/j.ccr.2010.11.010.

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