GSK-3β–Regulated N-Acetyltransferase 10 Is Involved in Colorectal Cancer Invasion

Hong Zhang, Wei Hou, Hua-Li Wang, Hai-Jing Liu, Xin-Ying Jia, Xing-Zheng Zheng, Yong-Xin Zou, Xin Li, Lin Hou, Michael A. McNutt, and Bo Zhang

Abstract

**Purpose:** NAT10 (N-acetyltransferase 10) is a nucleolar protein, but may show subcellular redistribution in colorectal carcinoma. In this study, we evaluated membranous staining of NAT10 in colorectal carcinoma and its clinical implications, and explored the mechanism of regulation of NAT10 redistribution.

**Experimental Design:** The expression and subcellular redistribution of NAT10, β-catenin, E-cadherin, and GSK-3β were evaluated by immunohistochemistry in 222 cases of colorectal carcinoma. Regulation of NAT10 and its influence on cell motility were analyzed with inhibitors of GSK-3β, transfection of wild-type or kinase-inactivated GSK-3β, or expression of various domains of NAT10, and evaluated with immunofluorescence, Western blotting, and Transwell assays.

**Results:** NAT10 localized mainly in the nucleoli of normal tissues, and was redistributed to the membrane in cancer cells, particularly at the invasive “leading edge” of the tumor. This correlated well with nuclear accumulation of β-catenin ($P < 0.001$; $\chi^2 = 68.213$). In addition, NAT10 membrane staining reflected the depth of invasion and tendency to metastasize (all $P$ values $< 0.001$), and was associated with a poorer prognosis ($P = 0.023$; $\chi^2 = 5.161$). Evaluation of the mechanism involved demonstrated that subcellular redistribution of NAT10 may result from its increased stability and nuclear export, which is brought about by inhibition of GSK-3β. Moreover, redistribution of NAT10 induces alteration of cytoskeletal dynamics and increases cancer cell motility.

**Conclusion:** The subcellular redistribution of NAT10 can be induced by decreases in GSK-3β activity. This redistribution increases cancer cell motility, and is thus, correlated with invasive potential and poorer clinical outcome. This finding suggests that NAT10 may be a useful prognostic marker and potential therapeutic target in colorectal carcinoma.

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be transactivated directly by (13, 14), and the cytoskeleton regulators Fascin and L1 can cytoskeletal remodeling involving actin or microtubules (EMT) in cancer cells (11, 12). Second, it may bring about naling can induce epithelial–mesenchymal transition through several pathways. First, the activation of Wnt sig-

NAT10 is a nucleolar protein, undergoes subcellular redistribution from the nucleolus in normal tissues to the cytoplasm and membrane in colorectal carcinoma cells. Membranous NAT10 staining correlates with nuclear translocation of β-catenin, and shows coincident distribution at the invasive front of cancer, and is also corre-

We found that N-acetyltransferase 10 (NAT10), which is a nucleolar protein, undergoes subcellular redistribution from the nucleolus in normal tissues to the cytoplasm and membrane in colorectal carcinoma cells. Membranous NAT10 staining correlates with nuclear translocation of β-catenin, and shows coincident distribution at the invasive front of cancer, and is also corre-

Patients and tissue specimens
A total of 222 surgically resected cases of colorectal carcinoma were collected from the archives of the Department of Pathology, Peking University Health Science Center, and the patients included 118 males and 104 females, with a median age of 64.4 years (age range from 25 to 99 years). None of the patients received preoperative chemo-

Materials and Methods

Staining evaluation
β-catenin staining was found to be membranous, cytoplas-

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Ethics statement
This study was approved by the Peking University Insti-
tutional Review Board and ethics committee before the start of the project.

Cell culture and treatment
HeLa, Lovo, and HCT116 cells were maintained in Dulbecco’s Modified Eagle Medium with high glucose (Gibco, Life Technologies) supplemented with 10% fetal bovine serum. Cells were incubated in a humidified atmosphere with 5% CO₂ at 37 °C. For cell treatment, varying concentrations of LiCl (Sigma), SB415286 (Sig-

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Plasmid construction and transfection

We cloned distinct fragments of the NAT10 gene into the eukaryotic expression vector pEGFP-C3, and obtained a series of GFP-NAT10s (GFP-NAT10-Fu, -NAT10-AN, and -NAT10-AC) plasmids as shown in Supplementary Table S1. Flag-tagged-NAT10, including full-length NAT10 (NAT10-Fu, 1–872 aa), HAT-deleted NAT10 (NAT10-AN, 549–872 aa), and C-terminus–deleted NAT10 (NAT10-AC, 1–849 aa), were constructed from the above GFP-NAT10s and inserted into the gw-assemble-zz plasmid, a kind gift from Dr. Wei Gu, Columbia University, New York, NY). Wild-type GSK-3β (GSK3β-WT) was generated by cloning RT-PCR amplified GSK-3β cDNA into the gw-assemble-zz plasmid, whereas kinase-inactivated GSK-3β (GSK3β-K85R) was constructed by site-directed mutagenesis of the GSK3β-WT plasmid. AN-β-catenin, the activated form of β-catenin was a kind gift from Dr. Walter Birchmeier (Max Delbrueck Center for Molecular Medicine, Berlin, Germany). All of the recombinant plasmids were confirmed by sequencing. Two small interfering RNAs were designed and synthesized as following: NAT10-KD1, 5’-CAGCACCACGUGCAGCAGAUAAGATT-3’; NAT10-KD2, 5’-GGCCCAAGCGUGUCCUGUGAAAATTT-3’; control, 5’-UCCUAAACGAUGUGGCUCGUUG-3’. Plasmids and synthesized siRNAs were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Immunofluorescent microscopy

Cells grown on cover slides were fixed in 4% formaldehyde (pH 7.4) for 15 minutes at room temperature, permeabilized with 0.1% Triton X-100 for 10 minutes or fixed in ice-cold methanol for 5 minutes. The cells were blocked with 1% horse serum for 60 minutes at room temperature, and incubated with primary antibody (diluted in blocking buffer) overnight. After three PBS washes, cells were incubated with fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC)–conjugated secondary antibodies (Sigma) for 60 minutes. Nuclei were counterstained with rhodamine isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC)–conjugated secondary antibodies. Images were observed and recorded using a fluorescence microscope (Model CX51; Olympus), and Photoshop version 7.0 (Adobe Systems Inc.) was used for image processing. For analysis of subcellular localization of NAT10, cells were treated with LiCl or SB415286 for 60 hours in culture and were directly incubated with antibody (1:200) at room temperature for 60 minutes, and then fixed 10 minutes by 4% formaldehyde and analyzed with a confocal microscope (Leica TCS SP8; Leica).

Statistical analysis

All analysis was performed using SPSS statistics software (Version 17.0, Chicago) and Excel 2007. Relationships between tumor markers and other parameters were studied using the χ² test, Fisher exact test, Continuity Correlation, or the independent t test when appropriate. The influence of NAT10 translocation on patient prognosis was analyzed on the basis of overall survival (OS) and disease-free survival (DFS). OS was defined as the time from initial diagnosis to death from any cause or last follow-up. DFS was estimated as the time from initial diagnosis to progression, recurrence, death, or last follow-up. Both DFS and OS curves were plotted using the Kaplan–Meier method and compared with log-rank tests. A P value of less than 0.05 was considered to be of statistical significance. All the statistical tests and P values were two-sided, and the level of significance was set at <0.05 (*), <0.01 (**), or <0.001 (**).
Figure 1. Subcellular staining of NAT10 correlated with activation of Wnt signaling in colorectal cancers. A, representative images of membranous staining of NAT10 and nuclear translocation of β-catenin in carcinoma. B, percentage of subcellular distribution of NAT10 and β-catenin in colorectal carcinoma. C, representative images of staining for NAT10 and β-catenin in carcinoma (a), high magnification of NAT10 and β-catenin stained in front zone of cancer invasion (b), (c), staining of NAT10 and β-catenin in infiltrating nests of cancer cells. D, survival curves based on membranous staining of NAT10 using the Kaplan–Meier method with the log-rank test. (a), membranous staining of NAT10 and patient DFS; (b), membranous staining of NAT10 and patient OS.
membranous staining (78.2%; 147 of 188; Supplementary Table S3). There was a positive statistical correlation between cytoplasmic or membranous NAT10 staining and nuclear accumulation of β-catenin (P < 0.001; Supplementary Table S3). It is noteworthy that the staining of membranous NAT10 and nuclear β-catenin was frequently coincidental at the invasive front or infiltrating nests of carcinoma (Fig. 1C). In addition, NAT10 and β-catenin immunostaining was also evaluated in metastatic colorectal carcinomas, which included 47 cases of lymphatic metastases and 7 cases of hepatic metastases. The metastatic colorectal carcinomas showed cytoplasmic staining of NAT10 in 98.1% (51 of 52), membranous staining in 84.6% (44 of 52), and nuclear staining in 82.7% (43 of 52; Supplementary Fig. S1). At the same time, nuclear staining of β-catenin was detected in 80.6% (42 of 52) of metastatic colorectal carcinomas, of which 97.2% (49 of 52) showed membranous staining, and all (52 of 52) were cytoplasmically positive (Supplementary Fig. S1).

Figure 2. Inhibition of GSK-3β induced cytoplasmic and membranous distribution of NAT10. A, HCT116 cells were treated with 20 mmol/L of LiCl or 20 μmol/L of SB (SB415286) for 12 or 24 hours and then subjected to immunofluorescent staining for anti-NAT10 and β-catenin. B, inhibition of GSK-3β increased levels of NAT10 in a dose-dependent manner. HCT116 cells were treated with indicated concentrations of LiCl for 24 hours, and cytoplasmic or nuclear proteins were extracted from whole cells. Levels of NAT10 and β-catenin in each fraction were measured by Western blotting. β-actin and lamin B1 were used as internal standards. C, long-term inhibition of GSK-3β induced membrane distribution of NAT10. Lovo cells were treated with 20 mmol/L LiCl for 60 hours and fixed with formaldehyde/Triton before double staining for NAT10 and β-catenin. D, detection of membranous NAT10 in living cells. HCT116 and Lovo cells were cultured in the presence of 20 mmol/L LiCl or 20 μmol/L SB415286 for 60 hours, respectively, and were further incubated with anti-NAT10 antibody for 1 hour and then fixed with formaldehyde. E, evaluation of membranous NAT10 by flow cytometry. HCT116 cells were cultured in the presence of 20 mmol/L LiCl or 20 μmol/L SB415286 for 24 hours (short term) or 60 hours (long term), respectively. After staining, NAT10 cells were analyzed by flow cytometry. NC, diluent treatment. All of the results are representative of at least three independent experiments.
As usual, a large portion of these cancers showed a loss of E-cadherin membranous staining (55.9%; 124 of 222), of which 76.6% (95 of 124) showed NAT10 staining, which was either cytoplasmic or membranous (Supplementary Fig S2). There was statistically significant correlation between loss of E-cadherin membrane staining and cytoplasmic or membranous NAT10 ($P = 0.008$, or $= 0.001$, respectively; Supplementary Table S4).

Figure 3. Activity of GSK-3β can regulate NAT10 levels. A, inhibition of proteasome enhanced the upregulation of NAT10 induced by the decreased activity of GSK-3β. Lovo cells were treated either with proteasome inhibitor MG132 (5 μmol/L) alone or with addition of 20 μmol/L SB (SB415286) for 12 hours. Cells were double stained for NAT10 and β-catenin (top). At the same time, levels of NAT10 and β-catenin were measured by Western blotting (bottom). NC, diluent treatment. B, effects of GSK-3β expression on levels of endogenous NAT10. Constructs, Flag-GSK3β-WT or Flag-GSK3β-K85R, were transfected into HCT116 cells for 48 hours, and cell extracts were prepared and analyzed by Western Blotting. C, effects of GSK-3β expression on the stability of cytoplasmic NAT10. Flag-GSK3β-WT or Flag-GSK3β-K85R was cotransfected with NAT10-ΔC into HCT116 cells for 48 hours, and ectopically introduced GSK-3β, NAT10-ΔC, and endogenous β-catenin were analyzed by Western blotting. All experiments were repeated at least three times.

Figure 4. Inhibition of GSK-3β promoted nuclear export of NAT10. A, LMB reduced cytoplasmic NAT10. Lovo cells were treated with 20 ng/mL LMB for 12 or 24 hours, and were double stained for NAT10 (green) and β-catenin (red). B, LMB antagonized nuclear export of NAT10 induced by inhibition of GSK-3β. HCT116 cells were treated with 20 mmol/L LiCl alone, or with addition of 20 ng/mL LMB for 12 or 24 hours, and were then double stained with NAT10 (green) and β-catenin (red). Treated cells were also subjected to Western blotting for analysis of NAT10 and acetylated tubulin (C). All the experiments were repeated three times independently.
GSK-3β staining in normal epithelial glands was usually weak, and two staining patterns were found in the clinical collection of cancers, which included complete loss of staining or focal overstaining (Supplementary Fig. S2). Complete loss of GSK-3β staining was seen in 144 of 222 (64.9%) samples, and 69 (69 of 222; 31.1%) cases showed focal overstaining, but no nuclear staining was found as has been reported earlier (ref. 22; Supplementary Table S4). Neither loss of expression nor overexpression of GSK-3β correlated with cytoplasmic or membranous NAT10 staining ($P = 0.795$, or 0.872, respectively; see Discussion).

Taken together, these results suggest that cytoplasmic or membrane NAT10 expression is closely correlated with nuclear accumulation of β-catenin or attenuation of E-cadherin staining.

**NAT10 membrane staining is correlated with aggressive clinical behavior in colorectal carcinoma**

As membranous NAT10 staining almost overlaps with cytoplasmic staining in colorectal carcinoma, and is more frequently detected at the invasive front of the cancer, correlation between membranous NAT10 staining with...
GSK-3β–Regulated N-Acetyltransferase 10 in Colorectal Carcinoma Invasion

Expression of NAT10 regulated migration and invasion of cancer cells. A, expression of different domains of NAT10 influenced migration and invasion of cancer cells. HeLa or HCT116 cells with transfection of full-length NAT10 (NAT10-Fu), HAT-deleted NAT10 (NAT10-ΔH), or C-terminus–deleted NAT10 (NAT10-ΔC) were subjected to Transwell analysis. B, knockdown of NAT10 reduced migration and invasive potential of cancer cells. HCT116 cells were transfected with NAT10 RNAi (NAT10-KD1, -KD2), or random RNAi (NC) and subjected to Transwell analysis at 48 hours posttransfection. Left, migration assay. Right, Matrigel invasion assay. **, P < 0.001. All experiments were repeated three times. C and D, expression of NAT10 promotes metastasis of colorectal cancer. HCT116 or Lovo cells transfected with blank vector (NC), full-length NAT10 (NAT10-Fu), or cytoplasmic distribution of NAT10 (NAT10-ΔC) were transplanted into mice by tail-vein injection, and after 4 weeks, lung metastasis was inspected by macro- or microscopy, respectively. The representative macroscopic images of metastases and microscopic images of metastatic cancer nests in lungs from HCT116-inoculated mice. D, quantification of metastatic formation in mouse transplantation. The lung metastases in each of these groups were counted, respectively, and the average of metastases per mouse was calculated and plotted. **, P < 0.001.

GSK-3β is a multifunctional serine/threonine kinase, which is an important regulator in Wnt signaling activation. Downregulation of GSK-3β increases the stability of β-catenin and consequently results in nuclear translocation. We assumed that regulation of NAT10 should be an event parallel to activation of β-catenin in Wnt signaling. Two GSK-3β inhibitors, LiCl of non-ATP competitor and SB415286 of ATP competitor, were used to mimic activation of Wnt signaling in HeLa, HCT116, or Lovo cells, respectively (23). With LiCl (20 mmol/L) or SB415286 (20 μmol/L) treatment for 12 or 24 hours, NAT10 staining increased time-dependently in both the cytoplasm and nucleus or nucleoli (Fig. 2A). At the same time, nuclear translocation of β-catenin was also observed under the treatment with these inhibitors (Fig. 2A). Western blot analysis showed that LiCl treatment increases the total NAT10 level in a dose-dependent manner (Fig. 2B). In addition, subcellular fractionation demonstrated that this increase in NAT10 was both nuclear and cytoplasmic, and at the same time, there was marked increase in both nuclear and cytoplasmic β-catenin (Fig. 2B). Cells were also subjected to cotreatment with LiCl and FH535, which is an inhibitor of β-catenin/TCF. There was an obvious decrease in nuclear β-catenin, but in contrast, NAT10 was still clearly elevated (Supplementary Fig. S4), suggesting that inhibition of GSK-3β may directly induce increases in NAT10 without the involvement of β-catenin.

More interestingly, with prolonged LiCl treatment (LiCl treatment up to 60 hours), NAT10 appeared on the cell membranes of HCT116 and Lovo cells (Fig. 2C and D). This induced membrane distribution of NAT10 was also confirmed with flow cytometry in the presence of LiCl (20 mmol/L) or SB415286 (20 μmol/L), respectively (Fig. 2E). To determine whether inhibition of GSK-3β–induced expression and redistribution of NAT10 occurs at the transcriptional or posttranscriptional level, real-time PCR was carried out and results showed no significant change in levels of NAT10 mRNA (data not shown), indicating that inhibition of GSK-3β influences NAT10 protein posttranscriptionally, such as through subcellular traffic or alteration of NAT10 stability.

GSK-3β activity regulates NAT10 stability

GSK-3β usually mediates degradation of target proteins via the phosphorylation coupled ubiquitin/proteasome pathway. Therefore, levels of NAT10 were analyzed under...
treatment with the proteasome inhibitor MG132 (5 μmol/L) alone or in the presence of GSK-3β inhibitors in HeLa, HCT116, or Lovo cells. Treatment with MG132 obviously increased NAT10 (Fig. 3A), whereas cotreatment with SB415286 further upregulated levels of NAT10 as evaluated with immunofluorescence (Fig. 3A, top). Western blot analysis also confirmed increase of NAT10 under treatment with MG132 or SB415286 singly or in combination (Fig. 3A, bottom). In addition, direct regulation of NAT10 by GSK-3β was confirmed by ectopic expression strategy. The over-expression of GSK-3β (GSK-3β-WT) reduced NAT10 levels, whereas kinase-inactivated GSK-3β (K85R mutation; ref. 24) failed to decrease NAT10 (Fig. 3B). Similarly, cotransfection of GSK-3β and NAT10ΔC (cytoplasmic form) resulted in greater reduction of NAT10ΔC, and at the same time, β-catenin was also reduced. Conversely, inactivation of GSK-3β increased both of these proteins (Fig. 3C). These results demonstrated that GSK-3β may phosphorylate NAT10 and, thereby, promote its degradation via proteasomes.

### Inhibition of GSK-3β promotes nuclear export of NAT10

The fact that inhibition of GSK-3 induced particularly an increase of NAT10 in the cytoplasm made us assume that NAT10 cytoplasmic distribution could be involved in nuclear–cytoplasmic shuttling. Through GFP-fused strategy, the lysine-enriched segment “KQSKKLKNRETKN” at 850–862, which is similar to the well-known classic nuclear localization signal (NLS; ref. 25), was found to be responsible for nuclear or nucleolar localization of NAT10 (Supplementary Table S1). When lysines were mutated into alanine, there was a markedly decreased in nuclear and nucleolar distribution of NAT10 (Supplementary Table S1), demonstrating that the 850–862 sequence acts as a NLS, which mediates nuclear import of NAT10.

For some of nuclear proteins, there is a nuclear export system in which the factor CRM1 recognizes a special protein sequence that brings about export, resulting in shuttling between the cytoplasm and nucleus, constituting a dynamic interchange. As expected, we found several putative nuclear export signals (NES) dispersed over NAT10 (data not shown). LMB, which is a specific inhibitor of CRM1, was then used to treat Lovo cells. In the presence of 20 ng/mL LMB, cytoplasmic NAT10 decreased, and extended treatment of LMB caused reduction of NAT10 in both the nucleus and cytoplasm (Fig. 4A). At the same time, cells treated with LiCl plus LMB showed an obvious decrease in NAT10 cytoplasmic distribution as compared with the LiCl only group, and these cells showed relatively a low abundance of NAT10 in the nucleus as well (Fig. 4B). In addition, these results were also confirmed by Western blotting (Fig. 4C). This suggested that interruption of NAT10 nuclear export inhibits its dynamic flux, which may induce decrease of NAT10.

These results indicate that inhibition of GSK-3β promotes nuclear export of NAT10 in a CRM1-dependent manner, and may also contribute to cytoplasmic or membranous accumulation of NAT10.

### Cytoplasmic retention of NAT10 increases cytoskeletal stability and remodeling

Previous studies have shown NAT10 can acetylate microtubules (20), and we, therefore, wondered whether the cytoplasmic retention of NAT10 could cause an increase in microtubule acetylation. Upon transfection with NAT10ΔC, an obvious increase of acetylated tubulins was associated with cytoplasmic accumulation of NAT10 (Fig. 5A and B). In addition, inhibition of GSK-3β also increased acetylated tubulins (Fig. 5A and B). At the same time, p120-catenin, which is a factor involved in regulation of microtubules and maintenance of E-cadherin membrane localization, was reduced (Fig. 5A and B).

In addition, actin remodeling of cells was also induced. With increasing cytoplasmic NAT10, cells stained with phalloidin showed a decrease in stress fibers, but cell protrusions such as filopodia or lamellipodia increased (Fig. 5C). At the same time, focal adhesions labeled with vinculin decreased (Fig. 5D). As expected, with knockdown of NAT10 by interfering RNA (RNAi; NAT10-KD1 and -KD2; Fig. 5E), cell protrusions were apparently reduced (Fig. 5F).

### The expression and redistribution of NAT10 regulates cancer cell migration and invasion

To determine whether NAT10 induced cytoskeleton remodeling would influence cancer cell migration and invasion, Transwell assays with or without Matrigel were carried out. Either upregulation by introduction of full-length NAT10 (NAT10-Fu), or alteration of cytoplasmic distribution of NAT10 (with NAT10-ΔC) enhanced migration or invasive capability of HCT116 cells, but HAT-deleted NAT10 (NAT10-ΔN) did not (Fig. 6A). In addition, knockdown of NAT10 by RNAi (NAT10-KD1 and -KD2) markedly reduced migration or invasion of HCT116 cells (Fig. 6B). Moreover, cell migration and invasion were inhibited by overexpression of GSK-3β in HCT116, but were promoted by kinase-inactivated GSK-3β (GSK-3β-K85R) (Supplementary Fig. S5).

To evaluate the effects of NAT10 expression on metastatic potential, HCT116, or Lovo cells with stable expression of NAT10-Fu, or NAT10-ΔC were inoculated into mice by tail-vein injection, and, lung metastasis was inspected after 4 weeks, respectively. For HCT116 cells, macroscopic metastasis of lung was observed in NAT10-Fu (2 of 6) or NAT10-ΔC (2 of 6) transfected cells but not in the blank vector group (0 of 6; Fig. 6C). Although in microscopic form, lung metastasis could be detected in 6 of 6 of both of the NAT10-Fu or NAT10-ΔC group, 3 of 6 in the blank vector group (Fig. 6C). The average number of lung metastases in the NAT10-Fu (6.0 per mouse) or NAT10-ΔC group (6.2 per mouse) was much higher than that of the blank vector (1.7 per mouse; Fig. 6D). Similarly, for Lovo cells, macro- or microscopic metastasis of lung in NAT10-Fu (1 of 6, 5 of 6) or NAT10-ΔC (2 of 6, 6...
of 6) cells were much more frequent than those in blank vector controls (0 of 6, 3 of 6). The average number of metastases of NAT10-Fu or NAT10-ΔC (4.8 per mouse, 5.3 per mouse, respectively) increased in comparison with the blank vector controls (1.5 per mouse; Fig. 6D). The results demonstrated that elevated expression of NAT10 and its cytoplasmic accumulation could promote metastasis of colorectal cancer cells.

Discussion
At the present, nuclear translocation of β-catenin is the most important event in activation of Wnt signaling, as nuclear β-catenin coupling with TCF/LEF activates a series of target genes and regulates cell proliferation, EMT transition, and migration (26–28). As the subcellular redistribution of NAT10 is closely correlated with nuclear accumulation of α-catenin, NAT10 can simply be considered a factor involved in the Wnt signaling cascade. Although transactivation of downstream genes by β-catenin has been demonstrated in the major pathway (canonical pathway) for activation of Wnt signaling, there may be other mechanisms such as GSK-3β involved. Nuclear translocation of β-catenin is also a result of inhibition of its degradation by GSK-3β, which plays an important role in the Wnt signaling cascade (29, 30). This point strongly implies an association between NAT10 and GSK-3β. It is, therefore, of interest that a putative sequence "TPDESLGPS" (70–79 aa) for phosphorylation of GSK-3β is located in the N-terminus of NAT10, but direct modification of this sequence by GSK-3β needs verification. It is, thus, possible that NAT10 is not a target gene of β-catenin, but is rather a parallel factor in the Wnt signaling network. Nevertheless, in this study, we found no statistical correlation between NAT10 and GSK-3β with immunohistochemical staining. This may be due to difficulty in defining the status of GSK-3β by immunohistochemistry, as at this time there is no established standard for evaluation of GSK-3β staining. Moreover, abnormal nuclear staining of GSK-3β, which is described in a previous study, was not found in this study (22).

The activation of Wnt signaling not only upregulates expression of target genes but also protein traffic. For instance, activation of Wnt signaling increases the level of β-catenin through prevention of its degradation, and also enhances its nuclear import (30). Similarly, inhibition of GSK-3β can upregulate androgen receptor (AR) and at the same time accelerate its nuclear export (31). Despite the fact NAT10 is a nuclear protein that localizes mainly in the nucleoli, it seems that there is a NAT10 flux between the nucleus and cytoplasm (Fig. 4A), as treatment with LMB reduces the NAT10 in both nuclear and cytoplasm pools (Fig. 4A and B). In addition, in view of the fact NAT10 can be regulated by GSK-3β, it is assumed that cytoplasmic NAT10 can be degraded by GSK-3β activity, and reduction of Wnt signaling activity results in the attenuation of NAT10 degradation. At the same time, there is increased nuclear export of NAT10 via an unknown mechanism, inducing the cytoplasmic or membranous redistribution of NAT10.

The metastatic potential of cancer cells is mediated by migratory apparatus, including filopodia, lamellipodia, and invadosomes formed through cytoskeletal remodeling (32, 33), which is generally regulated by the well-known RhoGTPase that induces actin remodeling via the regulators Rac1, CDC42, or RhoA (34, 35). Remodeling of the cytoskeleton is usually achieved by protein modification such as phosphorylation and acetylation/deacetylation (36). The cytoskeleton can also be acetylated or deacetylated by a group of catalytic proteins, such as p300/PCF, HDAC, or SirT1/2, and so on (37, 38). Acetylation of microtubules usually enhances their stability, whereas deacetylation of the cytoskeleton promotes dynamic activity (37–39). Although deacetylation of the cytoskeleton has been demonstrated to be associated with increase in the motility of cancer cells, some studies have shown that the balance of acetylation and deacetylation is more important for cell migration (40). Acetylation of either p120-catenin or GDIα can trigger RhoGTPase activity to induce actin remodeling via the regulators Rac1, CDC42, or RhoA (41, 42). In a previous study, we showed that NAT10 is able to acetylate microtubules (17). As such, it is possible that membrane-translocated NAT10 may directly acetylate membrane-associated microtubules, or possibly actin, to regulate cell motility. Further studies to evaluate this possibility are warranted.

The 5-years survival rate for patients with colorectal carcinoma is largely dependent on lymph node and organ metastasis, and there has been little success in developing effective therapeutic agents against metastatic colorectal carcinoma (43). We suggest that NAT10 could be used as a marker or target for predicting or preventing spread of colorectal cancer, thus improving prognosis of colorectal cancers. In addition, the specific membrane translocation of NAT10 can be used as a marker for capturing circulating tumor cells (CTC), which has been demonstrated to be a very important index for monitoring treatment and recurrence of colorectal cancer (44).

It has long been noted that a few of the nucleolar proteins, such as B23 (nucleophosmin), C23 (nucleolin), and Nopp140 can redistribute in the cell membrane, mediating a variety of cellular activities such as cytoskeleton assembly and angiogenesis, which are far removed from the originally described function of these molecules (45–50). The present study reveals a functional link between membrane distribution of a nucleolar protein and invasive neoplastic potential. This novel finding may provide impetus for further exploration of functional redistribution of nucleolar proteins.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: H. Zhang, H.-J. Liu, B. Zhang

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