Title page

GSK-3β-regulated N-acetyltransferase 10 Is Involved in Colorectal Cancer Invasion

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Statement of Translational Relevance
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 (CRC) cells. Membranous NAT10 staining correlates with nuclear translocation of β-catenin, and shows coincident distribution at the invasive front of cancer, and is also correlated with depth of tumor invasion, metastases and advanced CRC clinic stage. Cases showing positive membranous NAT10 staining therefore showed poorer clinical outcome in comparison with cases which are NAT10 membrane negative. Further study demonstrated that subcellular redistribution of NAT10 can result in increased stability and nuclear export of this molecule, which is brought about by inhibition of GSK-3β. Redistribution of NAT10 induced dynamic cytoskeletal changes and increased cancer cell migration and invasion. These findings show NAT 10 membrane staining reflects activation of Wnt-signaling, and NAT 10 may therefore be a prognostic marker for CRCs.

Abstract

**Purpose:** NAT10 (N-acetyltransferase 10) is a nucleolar protein, but may show subcellular redistribution in colorectal carcinoma (CRC). 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, 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 Trans-well 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, χ²=68.213). In addition, NAT10 membrane staining reflected depth of invasion and tendency to metastasize (all P values < 0.001), and was associated with poorer prognosis (P=0.023, χ²=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 suggests NAT10 may be a useful prognostic marker and potential therapeutic target in CRC.

**Introduction**

Colorectal carcinoma (CRC) is one of the most common causes of cancer related death in the world, and there is potential both for cancer recurrence and distant metastasis following surgical excision of the primary lesion (1,2). CRCs are genetically classified into hereditary and sporadic types, and deficient DNA mismatch repair (MMR) genes or mutation of APC (adenomatosis polyposis coli) tumor suppressor gene, which leads to activation of the Wnt/β-catenin signaling, has been identified to be the corresponding molecular events, respectively (3-6). However, in contrast to the well-established molecular mechanism of CRC tumorigenesis, much less is known
about the molecular basis for formation of metastases in CRC (2).

In the past decade, the association of CRC progression with the Wnt signaling pathway has been extensively documented. Wnt signaling plays a major role not only in cell proliferation, but also in the polarity, adhesion, and migration of colorectal epithelial cells (6,7). The canonical Wnt/β-catenin pathway has been well worked out, and β-catenin plays a pivotal role in Wnt-signaling, with dual function in epithelial cells. On the one hand, this molecule is a component of adhesion junctions that is essential for linking the cytoplasmic tail of E-cadherins to the cytoskeleton (6-8). A complex containing APC/Axin/GSK-3β (glycogen synthase kinase-3β) effectively degrades unbound, cytoplasmic β-catenin. In addition, upon activation of the Wnt-signaling pathway, β-catenin accumulates in the nucleus where it binds to the family of TCF/LEF factors and plays a second role involving transcription (8). In fact, nuclear accumulation of β-catenin is generally considered to be an indication of oncogenesis and tumor progression (9,10). In addition, activation of Wnt signaling may trigger cancer invasion and metastasis through several pathways. First, the activation of Wnt signaling can induce EMT (epithelial mesenchymal transition) in cancer cells (11,12). Second, it may bring about cytoskeletal remodeling involving actin or microtubules (13,14), and the cytoskeleton regulators Fascin and L1 can be transactivated directly by β-catenin (15,16). It has also been observed that nuclear accumulation of β-catenin and its target genes such as Fascin and L1 are usually distributed at the invasive front of the tumor rendering colorectal cancer patients susceptible to tumor recurrence and metastasis, and leading to unfavorable outcomes (15-17).

NAT10 (N-acetyltransferase 10) is a nucleolar protein with histone acetylation activity, and it has been found to be involved in regulation of telomerase activity, DNA damage response and cytokinesis (18-20). NAT10 consists of 872aa with an acetyltransferase domain spanning
376-450aa and a lysine-rich C-terminus (835-872aa). In the present study, we discovered sub-cellular redistribution of NAT10 in colorectal cancers, which correlates well with Wnt-signaling activity and reflects invasive potential. We showed redistribution of NAT10 is caused by decreased activity of GSK-3β, and is able to promote cell motility through inducing cytoskeleton dynamics.

Materials and Methods

Ethics statement

This study was approved by the Peking University Institutional Review Board and ethics committee prior to the start of the project.

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 chemotherapy. 4% buffered formalin fixed and paraffin-embedded sections (4 μm thick) were stained with hematoxylin and eosin for histologic evaluation of histological diagnosis, typing and grading. Age, gender, tumor size, depth of invasion, lymphatic invasion, distant organ metastases, TNM stage and modified Dukes’ stage (21) were obtained by review of medical charts and pathologic records. The immunostaining of DNA mismatch repair genes (MMR), including MLH1, MSH2 and MSH6, was positive in all of 222 cases, indicating their belonging to sporadic CRCs.

Among these CRC patients, 184 cases were carefully followed to determine postoperative survival. During follow-up, tumors recurred in 52 patients (28.3%), and sites of recurrence included liver (30 patients), colon (2), lung (7), brain (1), ovary and pelvis (2), lymph nodes (4), bone (2), and peritoneum (3), and in 1 case there was diffuse whole body tumor involvement. 38 patients (20.7%) were dead of disease by the end of the follow-up period.

Staining evaluation

β-catenin staining was found to be membranous, cytoplasmic, nuclear or some combination of these, and ≥10% cells with nuclear β-catenin staining was regarded as positive. NAT10 staining
showed nucleolar, nuclear, cytoplasmic, membranous, and combined patterns. Each pattern of subcellular staining was separately recorded, and positive staining in $\geq 10$ of cells was defined as positive. E-cadherin staining is normally membranous and thus E-cadherin expression was defined as negative when $\geq 10\%$ of tumor cells showed loss of membrane staining. GSK-3$\beta$ showed weak staining in the cytoplasm or membrane of normal glandular epithelia (defined as normal), but cancer cells were either negative staining (-), or showed focal over-staining (+). Nuclear staining of GSK-3$\beta$ was not observed, which differs from what was described in a previous report (22). Stained slides were reviewed separately by two independent reviewers blinded to the clinical data.

**Cell culture and treatment**

HeLa, Lovo, HCT116 cells were maintained in Dulbecco 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, St Louis, MO, USA), SB415286 (Sigma), NaCl (Chemical Inc, Beijing, China), FH535 (Calbiochem, Darmstadt, Germany), or Leptomycin B (LMB) (Beyotime, Jiangsu, China) were added to the medium for indicated times.

**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-$\Delta N$, -NAT10-$\Delta C$) plasmids as shown in Table S1. Flag-tagged-NAT10, including full length NAT10 (NAT10-Fu, 1-872aa), HAT-deleted NAT10 (NAT10-$\Delta N$, 549-872aa), and C-terminus-deleted NAT10 (NAT10-$\Delta C$, 1-849aa), 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, USA). Wild-type GSK-3$\beta$ (GSK3$\beta$-WT) was generated by cloning RT-PCR amplified GSK-3$\beta$ cDNA into the gw-assemble-zz plasmid, while kinase-inactivated GSK-3$\beta$ (GSK3$\beta$-K85R) was constructed by site-directed mutagenesis of the GSK3$\beta$-WT plasmid. $\Delta N$-catenin, the activation
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 NAT10-KD1: 5’-CAGCACCACUGCUGAGAAUAAGATT-3’; NAT10-KD2: 5’-GGCCAAAGCUGUCUUGAAATT-3’. Plasmids and synthesized siRNAs were transfected with LipofectaminTM-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 min at r. t., permeabilized with 0.1% Triton X-100 for 10 min or fixed in ice-cold methanol for 5 min. The cells were blocked with 1% horse serum for 60 min at r. t., 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 min. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) for 3 min, and mounted with anti-fade solution. Results were observed and recorded using a fluorescence microscope (Model CX51; Olympus, Tokyo, Japan), and Photoshop version 7.0 (Adobe Systems Inc.) was used for image processing. For analysis of cell surface NAT10, cells were treated with LiCl or SB415286 for 60h in culture and were directly incubated with antibody (1:200) at r. t. for 60 min, and then fixed 10 min by 4% formaldehyde and analyzed with a confocal microscope (Leica TCS SP8, Leica, Germany).

**Statistical analysis**

All analysis was performed using SPSS statistics software (Version 17.0, Chicago, IL, USA) and Excel 2007. Relationships between tumor markers and other parameters were studied using the chi-square test, Fisher’s exact test, Continuity Correlation or the independent t-test when appropriate. The influence of NAT10 translocation on patient prognosis was analyzed based on
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 statistically significance. All the statistical tests and P values were 2-sided, and
the level of significance was set at <0.05 (*), <0.01 (**), or <0.001 (***)

Results
Subcellular redistribution of NAT10 in colorectal cancers

NAT10 is expressed in various types of human cancer, where it shows nuclear/nucleolar staining
(20). In this study, NAT 10 immunostaining in tumor cells was generally diffuse, and much more
intense than staining in non-neoplastic glandular cells. More interestingly, NAT10 subcellular
staining was sharply altered in cancers in contrast to non-neoplastic tissues, in which staining was
mainly found in nucleoli of glandular cells with little nucleoplasmic staining, but without
cytoplasmic or membranous staining (Fig. 1A). Instead, only 29.7% (66/222) of CRCs showed
nucleolar staining, but most of cases showed nucleoplasmic staining (91.9%, 204/222).
Unexpectedly, more than half of CRCs also showed cytoplasmic staining (69.4%, 154/222), and at
the same time most of these even showed focal membranous staining with NAT10 (67.1%,
149/222) (Fig. 1A and B). It is noteworthy that membranous positive NAT 10 was usually found at
the invasive front tumor zone in infiltrating nests of cancers (Fig. 1C).

To determine whether this change in NAT10 distribution is a result of genomic mutation, 28
exons of the NAT10 gene were analyzed by PCR-direct sequencing (Table S2) with the colorectal
cancer cell lines HCT116 and Lovo, and 20 cases of CRC. However, no mutations were detected
in these cell lines and cancer specimens (data not shown).
These results indicated that NAT10 immunostaining in colorectal cancers is mainly due to its subcellular re-distribution which suggests involvement in carcinogenesis and cancer behaviors.

**Subcellular redistribution of NAT10 correlates with nuclear accumulation of β-catenin in colorectal cancers**

Since Wnt-signaling plays a pivotal role in sporadic colorectal carcinogenesis, the expressions of β-catenin, E-cadherin and GSK-3β were immunohistochemically detected, and their correlations with redistribution of NAT10 were also evaluated, respectively.

β-catenin immunostaining showed membrane staining in nearly all carcinomas to a varying extent (220/222), but a large proportion of these cases also showed cytoplasmic (80.2%, 178/222) and focal nuclear staining (84.7%, 188/222) (Fig. 1A and B). Almost two-third of cases with nuclear accumulation of β-catenin also showed either cytoplasmic NAT10 staining (79.3%, 149/188), or focal membranous staining (78.2%, 147/188) (Table S3). There was a positive statistical correlation between cytoplasmic or membranous NAT10 staining and nuclear accumulation of β-catenin (P<0.001) (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 CRCs which included 47 cases of lymphatic metastases and 7 cases of hepatic metastases. The metastatic CRCs showed cytoplasmic staining of NAT10 in 98.1% (51/52), membranous staining in 84.6% (44/52) and nuclear staining in 82.7% (43/52)(Fig. S1). At the same time, nuclear staining of β-catenin was detected in 80.6% (42/52) of metastatic CRCs, of which 97.2% (49/52) showed membranous staining, and all (52/52) were cytoplasmically positive (Fig. S1).

As usual, a large portion of these cancers showed a loss of E-cadherin membranous staining (55.9%, 124/222), of which 76.6% (95/124) showed NAT10 staining which was either cytoplasmic or membranous (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) (Table S4).

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 over-staining (Fig. S2). Complete loss of GSK-3β staining was seen in 144 of 222 (64.9%) samples, and 69 (69/222, 31.1%) cases showed focal over-staining, but no nuclear staining was found as has been reported described (22) (Table S4). Neither loss of expression nor over-expression 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 CRC, and is more frequently detected at the invasive front of the cancer, correlation between membranous NAT10 staining with clinicopathologic parameters, such as depth of invasion, metastasis, TNM and Duke’s staging were statistically analyzed (Table S5). NAT10 membrane positivity was found in 109 of 137 cases (79.6%) of advanced carcinoma (T3 and T4), while early carcinomas limited to the muscularis (≦T2) showed membrane staining in just 40/85 cases (47.1%) (P<0.001, χ²=25.109) (Table S5). 83.7% (87/104) of cases with lymphatic or distant organ metastases at diagnosis showed membrane expression of NAT10, but only 52.5% of cases (62/118) (P<0.001, χ²=24.244) without metastases showed NAT10 membrane expression (Table S5). In addition, there was a positive linear correlation between NAT10 membrane expression and TNM or Dukes’ stage.
As expected, there were similar results with analysis of correlation between NAT10 cytoplasmic staining and clinicopathologic features (data not shown).

Kaplan-Meier single-factor analysis and the log-rank test showed a statistically significant decrease in disease-free survival (DFS) in CRC patients with positive NAT10 membrane staining (Fig. 1D, a). Nevertheless, in evaluation of patient overall survival (OS), there was no significant difference in NAT10 membrane positive and negative cases (Fig. 1D, b).

These data indicate that NAT10 membrane staining is associated with a more aggressive CRC phenotype with higher metastatic potential and worse prognosis.

**Inhibition of GSK-3β increases the level of NAT10 and induces subcellular redistribution**

Redistribution of NAT10 reflects activation of Wnt-signaling in CRC, and in particular there is correlation of NAT10 redistribution with β-catenin nuclear translocation as shown above. This raised a question as to whether NAT10 is a characteristic target gene of β-catenin involved in Wnt-signaling activation. For analysis of this possibility, a constitutively active form of β-catenin (ΔN-β-catenin) was introduced into cells, and there was no evidence of NAT10 regulation by β-catenin found by immunofluorescence, or by Western blotting (data not shown). The transcriptional activity of the NAT10 promoter with co-transfection of β-catenin was evaluated with the luciferase assay and there was no increase in reporter activity (Fig. S3). The results argue that NAT10 is not a β-catenin target gene.

GSK-3β is a multifunctional serine/threonine kinase which is an important regulator in Wnt signaling activation. Down regulation 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 (20mM) or SB415286 (20μM) treatment for 12h or 24h, 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 LiCl treatment increases the total NAT10 level in a dose-dependent manner (Fig. 2B). In addition, subcellular fractionation demonstrated 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 co-treatment 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 (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 h), 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 (20mM) or SB415286 (20μM), respectively (Fig. 2E).

To determine whether inhibition of GSK-3β induced expression and redistribution of NAT10 occurs at the transcriptional or post-transcription level, real-time PCR was carried out and results showed no significant change in levels of NAT10 mRNA (data not shown), indicating inhibition of GSK-3β influence NAT10 protein post-transcriptionally, 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 (15mM) alone or in the presence of GSK-3β inhibitors in HeLa, HCT116 or Lovo cells. Treatment with MG132 obviously increased NAT10 (Fig. 3A), while co-treatment with SB415286 further up-regulated levels of NAT10 as evaluated with immunofluorescence (Fig. 3A, upper panels). Western Blot also confirmed increase of NAT10 under treatment with MG132 or SB415286 singly or in combination (Fig. 3A, lower panels). In addition, direct regulation of NAT10 by GSK-3β was confirmed by ectopic transfection strategy. The over-expression of GSK-3β (GSK-3β-WT) reduced NAT 10 levels, while kinase-inactivated GSK-3β (K85R mutation) (24) failed to decrease NAT10 (Fig. 3B). Similarly, co-transfection 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 assumed 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) (25), was found to be responsible for nuclear or nucleolar localization of NAT10, (Table S1). When lysines were mutated into alanine, there was a markedly decreased in nuclear and nucleolar distribution of NAT 10 (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 where the factor CRM1 recognizes a special protein sequence which 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). Leptomycin B (LMB) which is a specific inhibitor of CRM1 was then used to treat Lovo cells. In the presence of 20ng/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 a 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, B). In addition, inhibition of GSK-3β also increased acetylated tubulins (Fig. 5A, 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, B).

In addition, actin remodeling of cells was also induced. With increasing cytoplasmic NAT10, cells stained with phalloidin showed a decrease in stress fibres, 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 knock-down of NAT10 by interfering RNA (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 up-regulation 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-ΔAN) did not (Fig. 6A). In addition, knock-down 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 over-expression of GSK-3β in HCT116, but were promoted by kinase-inactivated GSK-3β (GSK-3β-K85R) (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 (2/6), or NAT10-ΔC (2/6) transfected cells but not in blank vector group (0/6) (Fig. 6C). While in microscopic, lung metastasis could be detected in 6/6 of both of NAT10-Fu, or NAT10-ΔC group, 3/6 in blank vector group (Fig. 6C). The average number of lung metastases in group of NAT10-Fu (6.0 per mouse), or NAT10-ΔC (6.2 per mouse) was much higher than that of blank vector (1.7 per mouse) (Fig. 6D). Similarly,
for Lovo cells, macro- or microscopic metastasis of lung in NAT10-Fu (1/6, 5/6), or NAT10-ΔC (2/6, 6/6) cells were much more frequent than those in blank vector controls (0/6, 3/6). The average number of metastases of NAT10-Fu, or NAT10-ΔC (4.8 per mouse, 5.3 per mouse, respectively) increased in comparison to 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 β-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, since 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 up-regulates 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 up-regulate androgen receptor (AR) and at the same time accelerate its nuclear export (31). Despite the fact NAT10 is a nuclear protein which localizes mainly in the nucleoli, it appears 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, 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 which 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, while 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 GDI α can trigger RhoGTPase activity to induce actin remodeling via the regulators Rac1, CDC42 or RhoA (41,42). In a previous study, we showed 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 CRC patients is largely dependent on lymph node and organ metastasis, and there has been little success in developing effective therapeutic agents against metastatic CRC (43). We suggest 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.

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Authors’ Contributions

Conception and design: B. Zhang, H. Zhang
Development of methodology: B. Zhang, H. Zhang, H. Wang, W. Hou
Acquisition of data (acquisition of patient samples and clinical data, provision of laboratory facilities.): B. Zhang, H. Zhang, H. Wang, W. Hou, X.-Y. Jia
Writing, review, and revision of the manuscript: B. Zhang, H. Zhang, Michael A McNutt
Administrative, technical, or material support: X.-Z. Zheng, X. Li, H.-J. Liu, L. Hou
Study supervision: B. Zhang

References


50. Kubler D. Ecto-protein kinase substrate p120 revealed as the cell-surface-expressed nucleolar

Figure Legends

**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 CRC. **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.

**Figure 2.** Inhibition of GSK-3β induced cytoplasmic and membranous distribution of NAT10. **A,** HCT116 cells were treated with 20mM LiCl or 20μM SB (SB415286) for 12 or 24 h 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 h, 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 mM LiCl for 60 h 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 20mM LiCl or 20μM SB415286 for 60 h, respectively, and were further incubated with anti-NAT10 antibody for 1 h and then fixed with formaldehyde. **E,** evaluation of membranous NAT10 by flow cytometry. HCT116 cells were cultured in the presence of 20mM LiCl (or 20μM SB415286) for 24 h (short term) or 60 h (long term), respectively. After staining NAT10 cells were analyzed by flow cytometry. NC: diluent treatment. All of results are representative of at least three independent experiments.

**Figure 3.** Activity of GSK-3β can regulate NAT10 levels **A,** inhibition of proteasome enhanced
up-regulation of NAT10 induced by decreased activity of GSK-3 β. Lovo cells were treated either with proteasome inhibitor MG132 (15mM) alone or with addition of 20μM SB (SB415286) for 12 h. Cells were double stained for NAT10 and β-catenin (upper panels). At the same time, levels of NAT10 and β-actin were measured by Western blotting (lower panels). NC: diluent treatment. B, effects of GSK-3β expression on levels of endogenous NAT10. Constructs Flag-GSK3β-WT or Flag-GSK3β-K85R was transfected into HCT116 cells for 48 h, 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 co-transfected with NAT10-ΔC into HCT116 cells for 48 h, 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, Leptomycin B (LMB) reduced cytoplasmic NAT10. Lovo cells were treated with 20 ng/ml LMB for 12 h or 24 h, 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 20mM LiCl alone, or with addition of 20 ng/ml LMB for 12 h or 24 h, 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.

**Figure 5.** Increased cytoplasmic distribution of NAT10 stimulated microtubule acetylation of and actin remodeling. A, cytoplasmic distribution of NAT10 increased acetylation of microtubules. HCT116 cells were treated with 20mM LiCl, or transfected with NAT10-ΔC, which concentrated in the cytoplasm, and were stained for NAT10 (LiCL treated group only) and acetylated-tubulin (upper panels) or p120-catenin (lower panels). Treated cells were simultaneously subjected to Western blotting in which acetylated-tubulin, p120-catenin and cytoplasmic NAT10-ΔC were evaluated as shown in B. C, cytoplasmic redistribution of NAT10 induced actin remodeling. Lovo cells were treated with 20mM LiCl or 20μM SB (SB415286) for 12 h, and were double stained for NAT10 and Phalloidin. D, cytoplasmic redistribution of NAT10 reduced cell focal adhesions. HCT116 cells were transfected with GFP-NAT10-F, GFP-NAT10-ΔN, or GFP-NAT10-ΔC for 24 h, and then stained for Vinculin. The arrows indicate focal adhesions and
dashed lines outline transfected cells. E, depletion of NAT10 with intervening RNA. Lovo cells were transfected with NAT10 RNAi, NAT10-KD1, -KD2 or control (NC), and levels of NAT10 were measured by Western blotting. F, knock-down of NAT10 decreased cell protrusions. Lovo cells with transfection of NAT10 RNAi were fixed with methanol and double stained by NAT10 and actin. All experiments were repeated at least three times.

**Figure 6.** 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-ΔN), or C-terminus-deleted NAT10 (NAT10-ΔC) were subjected to Transwell analysis. B, knock-down 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 h post-transfection. Left panels: migration assay. Right panels: matrigel invasion assay. * * * P<0.001. All experiments were repeated three times. C-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. C, the representative macroscopic images of metastases and microscopic images of metastatic cancer nests in lungs from HCT116-inoculated mice. D, quantitation of metastatic formation in mouse transplantation. The lung metastases in each of groups were counted, respectively, and the average of metastases per mouse was calculated and plotted. * P<0.05 , * * P<0.01 , * * * P<0.001.
Figure 1

A

NAT10

β-catenin

Normal

Carcinoma

125μm

B

Positive cases of NAT10 in each subcellular compartment (%)

<table>
<thead>
<tr>
<th>Subcellular Compartment</th>
<th>Normal</th>
<th>Carcinoma</th>
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<tbody>
<tr>
<td>Nucleolus</td>
<td>29.7%</td>
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<td>Nucleoplasm</td>
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<tr>
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<tr>
<td>Membrane</td>
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C

NAT10

β-catenin

300μm

150μm

300μm

D

Cumulative DFS

<table>
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<tr>
<th>DFS Status</th>
<th>NAT10 Membrane</th>
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<tbody>
<tr>
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<td>Membrane Positive</td>
</tr>
<tr>
<td>Negative</td>
<td>Membrane Negative</td>
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Cumulative DFS

Time (month)

P = 0.023*

P = 0.168
Figure 3

A

NAT10  β-catenin  DAPI
NC
SB
MG132
SB+MG132

B

vector  GSK3β-WT  GSK3β-K85R
NAT10
Flag
β-actin

C

NAT10-ΔC  GSK3β-WT  GSK3β-K85R
NAT10-ΔC+
IB: Flag
GSK-3β
IB: Flag
β-catenin
IB: β-catenin
β-actin
IB: β-actin
Figure 5

(A) NAT10, acetyl-tubulin, DAPI

(B) 

<table>
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<tr>
<th></th>
<th>Flag-NAT10ΔC</th>
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</tr>
<tr>
<td>acetyl-tubulin</td>
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</tr>
<tr>
<td>Flag</td>
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<td>+</td>
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<tr>
<td>β-actin</td>
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</table>

(C) NAT10, Phalloidin, DAPI

(D) GFP, Vinculin, DAPI

(E) 

<table>
<thead>
<tr>
<th></th>
<th>NAT10</th>
<th>β-actin</th>
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<tr>
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<td>NAT10-KD1</td>
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<tr>
<td>NAT10-KD2</td>
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(F) NAT10, actin, DAPI

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