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Genetic disruption of *ATAT1* causes RhoA downregulation through abnormal truncation of C/EBPβ

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Microtubule acetylation has been shown to regulate actin filament dynamics by modulating signaling pathways that control actin organization, although the precise mechanisms remain unknown. In this study, we found that the downregulation of microtubule acetylation via the disruption *ATAT1* (which encodes α-tubulin N-acetyltransferase 1) inhibited the expression of RhoA, a small GTPase involved in regulating the organization of actin filaments and the formation of stress fibers. Analysis of *RHOA* promoter and chromatin immunoprecipitation assays revealed that C/EBPβ is a major regulator of *RHOA* expression. Interestingly, the majority of C/EBPβ in *ATAT1* knockout (KO) cells was found in the nucleus as a 27-kDa fragment (referred to as C/EBPβ^{p27}) lacking the N-terminus of C/EBPβ. Overexpression of a gene encoding a C/EBPβ^{p27}-mimicking protein via an N-terminal deletion in C/EBPβ led to competitive binding with wild-type C/EBPβ at the C/EBPβ binding site in the *RHOA* promoter, resulting in a significant decrease of *RHOA* expression. We also found that cathepsin L (CTSL), which is overexpressed in *ATAT1* KO cells, is responsible for C/EBPβ^{p27} formation in the nucleus. Treatment with a CTSL inhibitor led to the restoration of *RHOA* expression by downregulation of C/EBPβ^{p27} and the invasive ability of *ATAT1* KO MDA-MB-231 breast cancer cells. Collectively, our findings suggest that the downregulation of microtubule acetylation associated with *ATAT1* deficiency suppresses *RHOA* expression by forming C/EBPβ^{p27} in the nucleus through CTSL. We propose that CTSL and C/EBPβ^{p27} may represent a novel therapeutic target for breast cancer treatment. [BMB Reports 2024; 57(6): 293-298]

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INTRODUCTION

Tissue homeostasis and function are maintained through a complex combination of cellular activities. These activities ensure the stability of the tissue, enable it to react to external factors, and allow it to carry out its unique roles. At the cellular level, these processes include cell adhesion, migration, proliferation, and differentiation, as well as the organization and regulation of the cytoskeleton and extracellular matrix (ECM) (1). The synchronization of actin and microtubule dynamics, in particular, is critical for cell adhesion and migration (2). Microtubules, for example, can control the localization and activity of actin-binding proteins by transporting them to specific locations within the cell (3). Actin filaments, in turn, can alter microtubule architecture and dynamics by exerting mechanical forces on microtubule growth and orientation (4). As a result, dysregulation of this cooperation can lead to a variety of diseases, including cancer and neurological disorders (5, 6).

Ras homolog family member A (RhoA) regulates actin dynamics during cell migration by driving retraction at the trailing edge and promoting membrane protrusions at the leading edge via processes such as membrane ruffling, lamellae formation, and membrane blebbing (7). Despite the understanding of RhoA's activation mechanism and its significance in cell migration, an understanding of the transcriptional regulation of RhoA in cancer cells remains elusive. While several transcription factors are known to regulate RhoA expression (8), they fail to comprehensively explain the diverse regulatory mechanisms observed in various cancer cells, particularly in terms of how microtubule acetylation in response to ECM stiffness impacts RhoA expression. Recent research has identified the CCAAT box within the *RHOA* promoter as a critical regulatory element, with CCAAT/enhancer-binding protein β (C/EBPβ), a well-known transcription factor in cancer progression, emerging as a likely candidate involved in *RHOA*'s transcriptional regulation (9). Since C/EBPβ is known to exist in various protein isoforms with varied transactivation potential (10), their interplay and response to microtubule acetylation, as well as their specific role in modulating *RHOA*'s transcriptional activity, are unexplored areas that require further investigation.

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We have previously reported that the genetic disruption of alpha-tubulin N-acetyltransferase 1 (α-TAT1), a major α-tubulin acetyltransferase, inhibits colorectal and breast cancers invasion through dysregulation of actin-related adherent junctions and focal adhesions (11, 12). However, the molecular mechanisms leading to the inhibition of actin-related signaling by *ATAT1* depletion has not been identified. In this study, we demonstrated that *ATAT1* knockout (KO) MDA-MB-231 cells reduced actin-dependent signaling through inhibition of RhoA expression. We also found that *ATAT1* KO cells generate a variant of C/EBPβ lacking the N-terminal deletion by cathepsin L (CTSL), which ultimately competes with normal C/EBPβ at the *RHOA* promoter and represses RhoA expression. Together, these results suggest that the introduction of the gene encoding nuclear CTSL or N-terminal deleted C/EBPβ may be a promising therapeutic target for microtubule acetylation-mediated invasive cancer.

RESULTS

Downregulation of RhoA by genetic disruption of *ATAT1*

Quantitative analysis of actin-related morphology in *ATAT1* KO MDA-MB-231 cells revealed a significant decrease in the size of vinculin-positive focal adhesions in the peripheral region and the length of stress fibers compared with control cells, indicating that microtubule acetylation is involved in the formation of cellular tension (Fig. 1A).

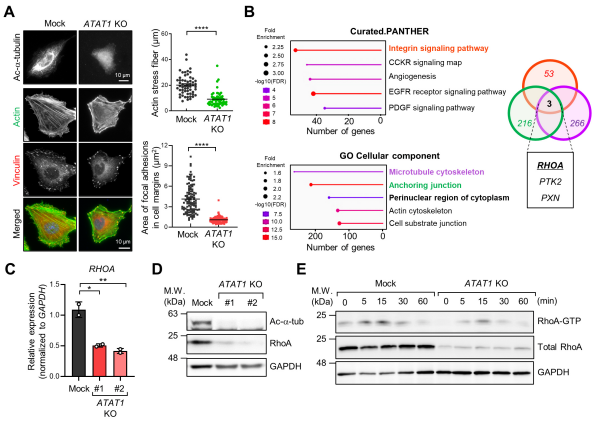

Fig. 1. Genetic disruption of *ATAT1* inhibits stress fiber and focal adhesion formation and reduces RhoA expression and activity. (A) Immunocytochemistry analysis of acetylated α-tubulin, focal adhesions and F-actin in mock and *ATAT1* KO cells. Scale bar, 10 μm. Graphs show the lengths of actin stress fibers between two focal adhesions and area of focal adhesions at the marginal region ($n > 100$). (B) Functional annotation of 3,644 genes downregulated in *ATAT1* KO cells using PANTHER and GO database and schematic diagram of the identification of the *RHOA* gene. (C) Results from qPCR analysis of the transcriptional expression of *RHOA* in mock and *ATAT1* KO (clones #1 and #2) MDA-MB-231 cells. (D) Immunoblotting analysis showing the protein level of RhoA in mock and *ATAT1* KO cells. (E) A RhoA activity assay was performed on mock and *ATAT1* KO cells. All error bars represent the standard deviation (SD) of the data.

To determine the underlying causes of the morphological changes observed in the *ATAT1* KO cells, we conducted functional analysis on differentially expressed genes (DEGs) using RNA-seq raw data obtained from mock and *ATAT1* KO cells. We performed a ShinyGO enrichment analysis (http://bioinformatics.sdstate.edu/go/) using 3,644 genes that were downregulated in *ATAT1* KO cells compared to wild-type (WT) cells, (fold change > 0.66 and adjusted P-values < 0.05). Fig. 1B shows the top 5 significantly downregulated categories according to the PANTHER database and Gene Ontology (GO) database for cellular components. Notably, *RHOA* was found to belong to all three categories, being most downregulated in *ATAT1* KO cells in the following area: microtubule cytoskeleton, anchoring junction and integrin signaling pathway (Fig. 1B).

To confirm RhoA expression in *ATAT1* KO cells, we examined the expression level of RhoA in both mock and *ATAT1* KO cells by RT-qPCR and Western blot analysis. Fig. 1C shows that significant reductions in *RHOA* transcript levels were observed in *ATAT1* KO cells compared with mock cells. Consistently, RhoA protein expression levels also decreased in *ATAT1* KO cells (Fig. 1D). Additionally, when the amount of active RhoA is normalized to GAPDH, it is evident that the absolute amount of intracellular active RhoA in *ATAT1* KO cells is up to three times lower than that in mock cells (Fig. 1E). These findings suggest that genetic disruption of *ATAT1* disrupts the formation of stress fibers and focal adhesions by inhibiting RhoA signaling.

Identification of C/EBPβ as a *RHOA* transcriptional activator

To explore the transcriptional regulation of *RHOA*, we analyzed the promoter region, up to 2 kb from the transcription start site, using the web-based program PROMO 3.0 and AliBaBa2.1. There were 140 cis-acting element sites predicted as candidates for potential competitive binders to the *RHOA* promoter region. Among them, we sorted out several binding proteins that met both the criteria of being reduced in *ATAT1* KO cells (fold change > 0.66, adjusted P-values < 0.05) relative to control cells and having a high hazard ratio (HR ≥ 1.0) in breast cancer patients (Supplementary Fig. 1A).

To ascertain the pivotal promoter region responsible for the transcriptional activation of *RHOA*, five progressive truncations of the *RHOA* promoter region were integrated into the pGL3 luciferase vector, as illustrated in Fig. 2A. The transcription efficacies of these varied *RHOA* promoter fragments (P1 to P5) were compared to that of pGL3-Basic vector in HEK293T cells. Notably, when the P2 construct (−1482 to +1) was introduced into the HEK293T cells, its transcriptional activity declined markedly compared with the P1 construct (−1972 to +1). Additionally, excising 381 bp from the P4 construct (−597 to +1) resulted in a significant decrease in luciferase activity, as seen in Fig. 2A. These results suggest the potential presence of

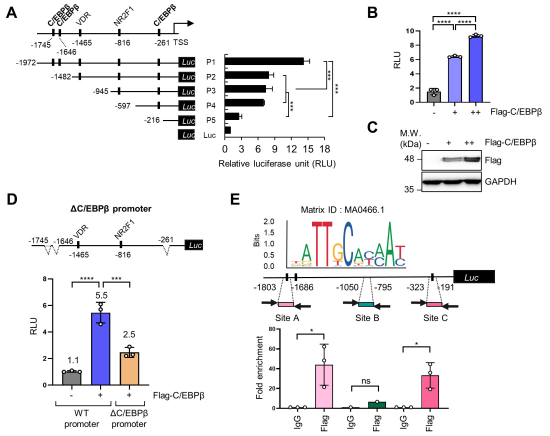

Fig. 2. C/EBPβ binds to the *RHOA* promoter and acts as an activator to increase *RHOA* expression. (A) Schematic representation of *RHOA* promoter-luciferase constructs and luciferase activity results. The schematic representation shows the one intact *RHOA* promoter (P1) and four truncated *RHOA* promoters from which the following regions had been deleted. (B) Luciferase assay results showing *RHOA* promoter activity according to the expression of C/EBPβ. (C) Immunoblotting analysis showing the protein level of Flag-C/EBPβ for samples used in (B). (D) Schematic representation of ΔC/EBPβ-*RHOA* promoter construct and luciferase assay results showing *RHOA* promoter activity according to the expression of C/EBPβ. (E) Results from ChIP analysis showing C/EBPβ binding to *RHOA* promoter. All data represent the means of independent experiments ± SD.

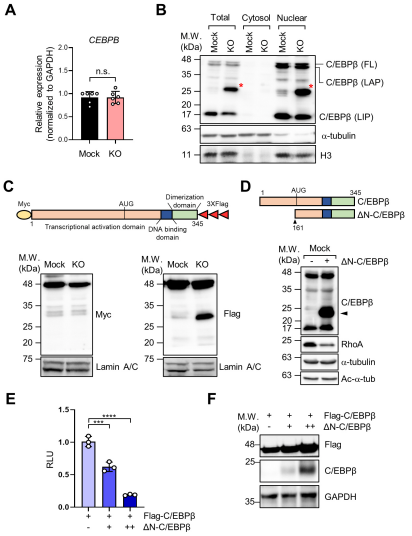

Fig. 3. C-terminal fragment of C/EBPβ found in *ATAT1* KO MDA-MB-231 cells causes a decrease in RhoA expression. (A) Results from qPCR analysis of the transcriptional expression of C/EBPβ in mock and *ATAT1* KO cells. (B) Immunoblotting analysis showing the protein level of C/EBPβ in the cytosolic and nuclear fractions of mock and *ATAT1* KO cell lines. Asterisk indicates the newly detected C/EBPβ band (C/EBPβ^{p27}). α-tubulin and H3 were used as markers of the cytoplasmic and nuclear factions, respectively. (C) Schematic representation of Myc-C/EBPβ-Flag construct and immunoblotting results using anti-Myc and anti-Flag antibodies. (D) Schematic representation of ΔN-C/EBPβ construct and immunoblot result showing the RhoA protein level according to the over-expression of ΔN-C/EBPβ in MDA-MB-231 cells. Arrowhead indicates ΔN-C/EBPβ band. (E) Luciferase assay results showing RhoA promoter activity according to the expression of C/EBPβ. (F) Immunoblotting analysis showing Flag-C/EBPβ and ΔN-C/EBPβ protein levels in the samples used in (E).

positive regulatory elements within the −1972 bp to −1482 bp and −597 bp to −261 bp regions.

Given that the two defined regions contain potential binding sites for C/EBPβ, we investigated the influence of C/EBPβ on *RHOA* promoter activity. To this end, we co-transfected C/EBPβ expression vector with pGL3-P1 promoter vector into HEK293T cells and subsequently assessed the ensuing luciferase activity. Remarkably, the transcriptional activity of the P1 promoter exhibited significant elevation (by 6 and 10 times, respectively) in a dose-dependent relationship with C/EBPβ expression, as shown in Fig. 2B, C. To further investigate whether the putative C/EBPβ binding site is indispensable for *RHOA* promoter activity, we constructed *RHOA* promoter variant (ΔC/EBPβ) that specifically deleted three putative C/EBPβ binding sites (−1745, −1646 and −261). As shown in Fig. 2D, following co-transfection with C/EBPβ expression vector, the transcriptional activity of the ΔC/EBPβ promoter was significantly attenuated relative to the native *RHOA* promoter. To corroborate this result, we performed chromatin immunoprecipitation (ChIP) assays to discern the possibility that C/EBPβ binds to the *RHOA* promoter region. The ChIP assay revealed that C/EBPβ preferentially binds with the −1808/−1686 and −323/−191 regions, while it appears to abstain from the −1050/−795 region (Fig. 2E). We also examined RhoA expression in cells treated with C/EBPβ shRNA to ascertain whether C/EBPβ regulates the expression of RhoA. Our findings demonstrated that the shRNA significantly reduced cellular RhoA protein expression (Supplementary Fig. 1B). Collectively, these results suggest that C/EBPβ functions as a potent transcriptional activator for *RHOA* transcription.

Reduced expression of *RHOA* by C/EBPβ lacking the N-terminus

To verify whether reduced expression of C/EBPβ in *ATAT1* KO cells downregulates *RHOA* expression, we confirmed RNA levels of C/EBPβ in mock and *ATAT1* KO cells. Contrary to the results obtained from the RNA sequencing analysis, the RNA and protein levels of C/EBPβ in *ATAT1* KO cells remained unchanged relative to the mock cells, as indicated in Fig. 3A, B. However, a notable increase was observed in a band of novel size—approximately 27 kDa—which was identified using a C/EBPβ-specific antibody in the *ATAT1* KO cells (Fig. 3B). Additionally, the ChIP assay using C/EBPβ antibody also showed that more C/EBPβ protein was bound to the *RHOA* promoter in *ATAT1* KO cells than in mock cells (Supplementary Fig. 2A). Given that C/EBPβ undergoes fragmentary processing du-

ring protein expression (13), these results raised the possibility that a 27-kDa protein detected with a C/EBPβ antibody exclusively in *ATAT1* KO cells could be a novel derivative with the ability to bind to the C/EBPβ *cis*-acting element.

To test this hypothesis, we first determined whether the 27-kDa protein originated from the C/EBPβ protein. Transfection with shRNA targeting C/EBPβ not only reduced the levels of the WT C/EBPβ protein—including well-known derivatives of C/EBPβ (liver-enriched activator protein [LAP] and liver-enriched inhibitory protein [LIP])—but also diminished the levels of the 27-kDa protein, which is predominantly found in the nuclear fraction of *ATAT1* KO cells (Supplementary Fig. 2B). Next, to determine whether the 27-kDa protein represents a fragment commonly produced through alternative translation or protein processing of C/EBPβ, such as the LIP fragment, we tagged the N-terminal and C-terminal ends of the C/EBPβ protein with Myc and Flag tags, respectively (Fig. 3C). We then assessed whether the 27-kDa band was included in the N- or C-terminus of C/EBPβ. Interestingly, the 27-kDa band was absent in the Western blot probed with the Myc antibody but was evident in the blot probed with the Flag antibody in *ATAT1* KO cells (Fig. 3C). Collectively, these results indicate that the 27-kDa protein, detected using C/EBPβ antibody predominantly in the nuclei of *ATAT1* KO cells, is a protein missing the N-terminus of C/EBPβ (hereafter referred to as C/EBPβ^{p27}).

Based on the molecular weight of C/EBPβ^{p27}, it is likely that it possesses a partial deletion of the transcriptional activation domain in the N-terminal region of C/EBPβ while retaining an intact DNA binding domain in the C-terminus. Consequently, C/EBPβ^{p27} likely exerts a negative regulatory effect upon binding to the C/EBPβ *cis*-acting element within the cell. To test this possibility, we engineered a truncated C/EBPβ fragment corresponding to approximately 27 kDa that contained both the intact DNA-binding and dimerization domains (referred to as △N-C/EBPβ; shown in Fig. 3D). Ectopic expression of △N-C/EBPβ significantly reduced the expression of RhoA in MDA-MB-231 cells (Fig. 3D). Additionally, we performed a pGL3-*RHOA* promoter activity assay in HEK293T cells after co-expression of △N-C/EBPβ and WT C/EBPβ vectors. As shown in Fig 3E, F, the activity of the *RHOA* promoter, which is dependent on WT C/EBPβ, decreased proportionately to the △N-C/EBPβ levels. These results suggest that C/EBPβ^{p27} competes with native C/EBPβ for binding at the *RHOA* promoter, ultimately acting to inhibit the transcriptional expression of *RHOA*.

C/EBPβ^{p27} formation by nuclear CTSL in *ATAT1* KO cells

To elucidate how *ATAT1* KO MDA-MB-231 cells produce C/EBPβ^{p27}, we used RNA sequencing data to examine the expression levels of various proteinases that could target nuclear C/EBPβ. Among the proteases, CTSL expression was significantly increased (5-fold) in *ATAT1* KO cells relative to the expression in mock cells, both at the transcript and protein levels (Fig. 4A, B). Notably, CTSL was discernible in the nuclear fraction of *ATAT1* KO cells (Fig. 4C).

To determine whether CTSL plays a role in the production of C/EBPβ^{p27}, we treated *ATAT1* KO cells with a CTSL inhibitor and then analyzed the C/EBPβ^{p27} expression pattern. Following this treatment, there was a significant decrease in C/EBPβ^{p27} and an increase in native C/EBPβ (Fig. 4D). CTSL inhibitor treatment also restored *RHOA* expression in *ATAT1* KO cells (Fig. 4E). The findings from the 3D invasion analysis indicated that the invasive ability of *ATAT1* KO cells was restored after CTSL inhibitor treatment (Fig. 4F). Collectively, these results suggest that abnormal CTSL induction in *ATAT1* KO cells pro-

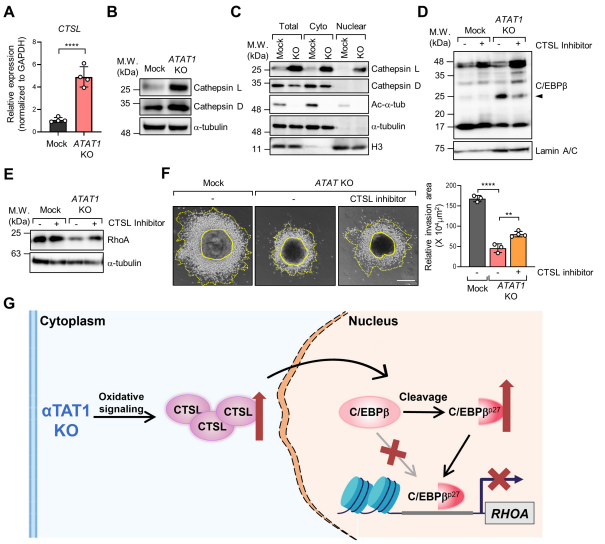

Fig. 4. Increased cathepsin L in the nuclei of *ATAT1* KO MDA-MB-231 cells inhibits cancer cell invasion by inducing the production of C/EBPβ C-terminal fragments. (A) Results from qPCR analysis of the transcriptional expression of CTSL in mock and *ATAT1* KO cells. (B) Immunoblotting analysis showing the protein levels of cathepsin L and cathepsin D in mock and *ATAT1* KO cells. (C) Immunoblotting analysis showing the protein levels of cathepsin L, cathepsin D, and Ac-α-tubulin in the different cellular fractions (total, cytosol and nuclear) of mock and *ATAT1* KO cell lines; α-tubulin and H3 were used as cytosolic and nuclear markers, respectively. (D, E) Immunoblotting analysis showing the protein levels of C/EBPβ and RhoA following the pharmacological inhibition of CTSL in mock and *ATAT1* KO cell lines. The nuclear fraction was used for analyzing the expression of C/EBPβ, and the level of lamin A/C was used as an internal control (D). Arrowhead indicates C/EBPβ^{p27}. The total fraction used for analyzing the expression of RhoA and α-tubulin was analyzed as a loading control (E). (F) 3D cell spheroid invasion assay of *ATAT1* KO cells with or without CTSL inhibitor. The yellow solid line represents the spheroid size on day 0. The yellow dotted line indicates invading cells observed on day 4 under each condition. Scale bar, 250 μm. The quantitative analysis results for the relative invasion area are expressed in the graph on the right. Data represent the means of independent triplicate experiments ± SD. (G) Graphical summary of the molecular mechanism involving the inhibition of RhoA expression.

motes C/EBPβ^{p27} formation, which in turn suppresses cancer cell mobility by downregulating *RHOA* expression (Fig. 4G).

DISCUSSION

Many diseases are associated with increased ECM stiffness. RhoA plays a crucial role in actin-dependent signaling and becomes abnormally active in cells responding to alternations in ECM stiffness (14). For these reasons, RhoA has been considered an attractive target for cancer interventions. However, extensive efforts have been made to develop inhibitors targeting Rho GTPases themselves, none have achieved clinical utility. Fig. 4G summarizes the results of our studies. we identified a series of molecular mechanisms involved in the inhibition of RhoA expression. First, we found that C/EBPβ is a transcription factor for the *RHOA* gene. Second, CSTL can truncate the N-terminus of C/EBPβ in the nuclei of *ATAT1*-depleted cancer cells to form C/EBPβ^{p27}, a 27-kDa variant. This variant functions as a negative regulatory factor for native C/EBPβ, leading to the inhibition of *RHOA* gene expression. These results indicate that CSTL, C/EBPβ, and *ATAT1* could serve as promising therapeutic targets for ECM stiffening and/or RhoA-dependent cancer treatment.

C/EBPβ is a well-known transcription factor required for tumor progression (10). It is processed into three proteins from a single transcript: LAP1, LAP2, and LIP (13). Interestingly, the LAP2 isoform is a more powerful transcriptional activator than full-length LAP1 (15). The LIP isoform, given its absence of an N-terminal activation domain, acts to inhibit the transcriptional activity of other C/EBP proteins, either through competing for C/EBP consensus binding sites or by forming inactivating heterodimers with other C/EBPs, thereby serving as a dominant negative (13). The C/EBPβ^{p27} investigated in this research shares similarities with LIP in that it lacks the N-terminal transcriptional activation domain. This protein subtype also competes with the native LAP2 protein, inhibiting its binding to the RhoA promoter, thus suppressing its transcriptional activity (Fig. 3E, F). Though the *in vivo* existence of C/EBPβ^{p27} remains unconfirmed, our findings hint at its potential role as a novel C/EBPβ-dependent gene regulator in certain cancers, like triple-negative breast cancer. Thus, these findings emphasize the potential significance of studying the mechanisms that promote C/EBPβ^{p27} generation in cancer cells. Such insights could pave the way for developing novel therapeutic agents that target C/EBPβ-dependent cancer cell activities, particularly those associated with breast cancer.

CTSL, a member of the cathepsin family, is essential for protein degradation within lysosomes, and it promotes cancer cell migration and invasion via ECM breakdown (16). Its ectopic expression is especially problematic, as it frequently correlates with a poor prognosis (17). Recent findings highlight an intriguing short CTSL isoform without a signal peptide that regulates gene expression by processing the CCAAT-displacement protein/cut homeobox transcription factor (18). In *ATAT1* KO cells, we found an increase in the mature CTSL form (25 kDa) in the nucleus, but nuclear CTSL (35 kDa) remained unaffected (Supplementary Fig. 3). The presence of mature CTSL in the nuclei of *ATAT1* KO cells raised intriguing questions about the cellular trafficking mechanism of CTSL. While CTSL is typically known to be in lysosomes, our results suggest that mature CTSL also could exist in nuclei and play a critical role in regulating cancer cell progression. One possible explanation lies in the dynamics of acetylated microtubules. In the context of *ATAT1* KO cells, there is a decrease in microtubule acetylation, which weakens the binding affinity between these acetylated microtubules and motor proteins such as kinesins (19). Under normal conditions, this interaction facilitates the transportation of intracellular vesicles, including in the contexts of endocytosis and exocytosis. However, given the decreased binding in *ATAT1* KO cells, CTSL's typical egression processes may be disrupted, making it less capable of escaping the cellular region. As a result, there is a propensity for CTSL to accumulate within the nucleus, likely facilitated by simple diffusion through the nuclear pore complex. This novel localization pattern underscores the multifaceted roles CTSL might play in cellular contexts where microtubule dynamics are altered, such as in *ATAT1* KO cells.

Each of α-TAT1, RhoA, C/EBPβ, and CTSL is already well known as an important protein in cancer processing. Bioinformatic analysis using Oncomine database of breast cancer patients showed that the expression of α-TAT1, RhoA and C/EBPβ had a strong clinical relevance to the progression of breast cancer (Supplementary Fig. 4). Here, we showed that α-TAT1, RhoA, C/EBPβ, and CTSL interact with each other to affect each other's activity and transcriptional regulation. The results suggest that the interactions of these proteins may affect cancer development differently from each individual function. This will be of great help in predicting the potential side effects that may arise from regulating the expression of these proteins, which are widely recognized as anticancer targets, on the development and behavior of cancer cells. Our results were observed in the limited context of the *ATAT1* KO MDA-MB-231 cell line. Therefore, additional research involving other breast cancer cell lines or animal models is essential for further validation. Additionally, our hypothesis about the regulation of CTSL activity by microtubule acetylation needs to be addressed through further research. Nevertheless, our results clearly showed that the downregulation of microtubule acetylation by *ATAT1* deficiency suppresses *RHOA* expression by forming nuclear C/EBPβ^{p27} through increased CTSL expression, resulting in a loss of motility in MDA-MB-231 cancer cells. The results of this study collectively suggest that regulating RhoA expression via the regulation of *ATAT1* activity or through the overexpression of the C/EBPβ^{p27}could represent a novel therapeutic strategy for combating breast cancer.

MATERIALS AND METHODS

Materials and methods are available in the supplemental material.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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