



The ubiquitin ligase RNF8 regulates Rho GTPases and promotes cytoskeletal changes and motility in triple-negative breast cancer cells

Bruno Pereira De Carvalho¹, Yi-Jye Chern¹ , Jiabei He¹ and Chia-Hsin Chan^{1,2} 

¹ Department of Pharmacological Sciences, Stony Brook University, NY, USA

² Stony Brook Cancer Center, Stony Brook University, NY, USA

Correspondence

C. -H. Chan, Department of Pharmacological Sciences, Basic Science Tower 8-121, Stony Brook University, Stony Brook, NY 11794-8651, USA

Tel: 631-444-3085 (Office); 631-444-9743 (Lab)

E-mail: chia-hsin.chan@stonybrook.edu

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The ubiquitin ligase RNF8 is known to induce epithelial-to-mesenchymal (EMT) transition and metastasis in triple-negative breast cancer (TNBC). Besides EMT, Rho GTPases have been shown as key regulators in metastasis. In this study, we investigated the role of RNF8 in regulating Rho GTPases and cell motility. We find that RNF8 knockdown in TNBC cells attenuates the protein and mRNA levels of Ras homolog family member A (RHOA) and cell division cycle 42 (CDC42). We show that the formation of filopodia, focal adhesions, and the association of focal adhesions to stress fibers is impaired upon RNF8 knockdown. Cell migration is significantly inhibited by RNF8 knockdown. Our study suggests a potential novel role for RNF8 in mediating cell migration in TNBC through regulation of the Rho GTPases RHOA and CDC42.

Keywords: CDC42; cell migration; cytoskeleton rearrangements; filopodia; focal adhesion; RHOA; RNF8; triple-negative breast cancer

Triple-negative breast cancer (TNBC) accounts for 15–20% of breast cancer. It is defined by the absence of estrogen and progesterone receptors and the lack of amplification/ overexpression of human epidermal growth factor receptor 2 [1]. Compared to other subtypes of breast cancer, TNBC has a higher rate of metastatic spread and poorer prognosis [1,2]. As about 90% of cancer-related deaths are caused by metastatic disease, it is crucial to understand the mechanisms of metastasis in order to improve the therapeutic outcomes of TNBC [3].

The Rho family of GTPases (Rho GTPases) have emerged as key regulators in metastasis [3,4]. Physiologically, Rho GTPases cycle between inactive (GDP-bound) and active (GTP-bound) states. This process is tightly regulated by guanine-nucleotide-exchange factors (GEFs) and GTPase-activating proteins (GAPs)

to control cell behaviors such as cell migration [4,5]. Rho GTPases are often deregulated in cancer cells, leading to the increased cell motility and consequent metastatic diseases. Cell migration is orchestrated by a series of actin cytoskeleton reorganization, which results in the formation of lamellipodia, filopodia, and stress fibers [6]. Stress fibers are large bundles of filamentous actin (F-actin) that are frequently anchored at one or both ends by focal adhesions, the membrane-associated macromolecular assemblies that engage with the surrounding extracellular matrix (ECM) *via* integrin receptors [7]. Focal adhesions linked to contractile stress fibers generate traction forces to extend the cell membrane protrusions and move the cell forwards [8]. Lamellipodia and filopodia are these membrane protrusions existing at the leading edge of motile cells [9]. Lamellipodia, loosely

Abbreviations

CDC42, cell division cycle 42; EMT, epithelial-to-mesenchymal; ER α , estrogen receptor α ; GAPs, GTPase-activating proteins; GEFs, guanine-nucleotide-exchange factors; LUC, luciferase; RHOA, Ras homolog family member A; TNBC, triple-negative breast cancer.

organized meshwork composed of branched actin filaments, seems to provide persistent protrusion over a surface for migrating cells [9]. Filopodia, on the other hand, contain unbranched, parallel bundles of actin fibers, exploring the environmental cues to guide cell migration [9,10].

Ras homolog family member A (RHOA), cell division cycle 42 (CDC42), and Rac family small GTPase 1 (RAC1) are the best-characterized members of the Rho GTPase family and have also been shown to be implicated in tumor progression and metastasis [4,11,12]. RHOA plays critical roles in the formation of focal adhesions as well as the assembly and contractility of stress fibers [6]. RHOA-induced stress fibers can anchor to focal adhesions and turn these adhesions into a connection between the ECM and the actin cytoskeleton. This ECM-actin connection allows the cell to gain traction during cell migration [13,14]. On the other hand, CDC42 and RAC1 are the major regulators of filopodia and lamellipodia, respectively [4]. Importantly, the overexpression of RHOA, CDC42, and RAC1 has been reported in various human tumors such as breast cancer, indicating their important roles in tumor progression [15–18]. While the mechanisms by which these three GTPases modulate cell motility and migration have been well characterized, the upstream mechanisms mediating the upregulation of these Rho GTPases in tumors are less understood and need further elucidation.

RNF8 ubiquitin E3 ligase was initially identified to be implicated in DNA damage signaling [19–21]. In addition to its role in DNA damage response, RNF8 was recently revealed to be involved in the tumor progression of breast, bladder, and colon cancers [22–26]. For example, Lee *et al.* and Kuang *et al.* independently reported the upregulation of RNF8 in breast cancer and its involvement in EMT transition and metastasis [22,24]. Lee *et al.* further demonstrated that RNF8 promotes EMT *via* K63-linked ubiquitination and subsequent activation and stabilization of TWIST protein, leading to cancer metastasis [22,23]. Kuang *et al.* [24] showed that RNF8-mediated metastasis may be associated with the accumulation of β -catenin and the activation of EMT-related gene transcription. RNF8 was also found to be a critical interacting partner of estrogen receptor α (ER α) to facilitate the ER α -mediated gene transactivation in the ER α -positive breast cancer [27]. RNF8 has also been shown to promote tumorigenesis in brain tumor by mediating K48-linked ubiquitination and the subsequent degradation of histone H3 [28]. Taken together, these studies suggest an emerging but critical role of RNF8 in tumor progression, highlighting the need to understand the

mechanisms by which RNF8 regulates these oncogenic processes.

Although RNF8 has been shown to be involved in the process of EMT and metastasis [22,24], the underlying molecular mechanism remains unclear. In this study, we investigated the role of RNF8 in Rho GTPases and showed that RNF8 regulates the expression of RHOA and CDC42 in TNBC cells. Surprisingly, we found that this RNF8-mediated protein expression change in RHOA and CDC42 is not associated with protein degradation, suggesting regulation at the transcriptional level may be involved. RNF8 depletion leads to the reduced formation of filopodia, focal adhesion, and stress fibers, leading to the reduction in cell motility. These findings suggest a new role of RNF8 in modulating the cytoskeletal dynamics, providing a novel insight into the mechanisms of metastasis in TNBC.

Materials and methods

Cell culture and reagents

BT549, MDA-MB-231, and 293T cells (from ATCC, Manassas, VA, US) were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin and maintained in a humidified atmosphere of 5% CO₂ at 37 °C. Cells were authenticated by short tandem repeat profiling by ATCC and routinely verified to be free of mycoplasma contamination *via* the R&D (Minneapolis, MN, US) MycoProbe[®] Mycoplasma Detection Kit. MG132 was purchased from EMD Millipore (Burlington, MA, US). To generate shRNA-resistant Xp-RNF8, silent mutations were introduced into the shRNF8 target sequence (TGGAGCAACTAGAG AAGACTT) by using the oligonucleotide 5'-cagcagcagg caagagtggagcagctgaaaagactttccaggaaggaac-3' (forward); and 5'-gttctcttctctgaaaagcttttcaagctgctccactctgctgctg-3' (reverse).

Viral infection

293T cells were co-transfected with envelope plasmid (VSV-G), packing plasmid (deltaVPR8.9), and RNF8 or luciferase (LUC) shRNA using calcium phosphate transfection method. RNF8-lentiviral shRNA-1 (5'-TGGAGCAACTA GAGAAGACTT-3') and RNF8 lentiviral shRNA-2 (5'-CCAAAGAATGACCAAATGATA-3') were transfected along with the packing and envelope plasmids. The virus particles were collected 48 h post-transfection and used to infect the host mammalian cells. The infected cells were then cultured in medium containing the appropriate antibiotics for selection.

Immunoblot analysis

Cells were harvested, and the cell lysates were obtained using RIPA buffer containing a protease inhibitor cocktail (Roche, Indianapolis, IN, US). Whole-cell lysates were separated by SDS/PAGE, transferred to polyvinylidene fluoride membranes, and probed with primary antibodies overnight and appropriate HRP-conjugated secondary antibodies for 45 min. Antibodies used for immunoblotting were as follows: anti-RAC1 (1 : 10 000; Millipore, clone 23A8), RHOA (1 : 5000; Santa Cruz, Dallas, TX, US, clone 26C4), CDC42 (1 : 1000; Santa Cruz, clone B-8), RNF8 (1 : 2000; Santa Cruz, clone B2), β -actin (1 : 20 000; Sigma, St. Louis, MO, US, clone AC-15), and GAPDH (1 : 10 000; Santa Cruz, sc-48167).

Real-time PCR

Total RNA was isolated from cells using TRIzol reagent (Qiagen, Hilden, Germany). Isolated total RNA was used for cDNA synthesis using the SuperScript™ II Reverse Transcriptase Kit (Life Science), and the synthesized cDNA was used to perform real-time PCR analysis using the SYBR Green Fast Master Mix (Applied Biosystems, Foster City, CA, US) on a StepOnePlus Real-Time PCR System (Applied Biosystems). The relative expression levels of mRNAs were quantified by $2^{-\Delta\Delta C_t}$ method. The primer sequences used for amplifications are as follows: RNF8, 5'-GCCCCATTTGTGCGGAAGGACAT-3' (forward) and 5'-AGCAGCGGAGTCACGGCTAGA-3' (reverse); RHOA, 5'-GAGCACACAAGGCGGGAG-3' (forward) and 5'-CTTGCAGAGCAGCTCTCGTAG-3' (reverse); CDC42, 5'-GACAGATTACGACCGCTGAGT-3' (forward) and 5'-TCA GACCTCTCTGTGTAAGTGC-3' (reverse); and GAPDH, 5'-GATTCCACCCATGGCAAATTC-3' (forward) and 5'-CTTCTCCATGGTGGTGAAGAC-3' (reverse). The gene expression levels were normalized to GAPDH.

Immunofluorescence

Coverslips were coated with $10 \mu\text{g}\cdot\text{mL}^{-1}$ of fibronectin (Sigma) for 1 h at 37 °C. The coated coverslips were washed three times with PBS and blocked with 1.5% BSA in PBS for 90 min at 37 °C. Cells were seeded upon coverslips in a 24-well plate. The confluency of cells on the coverslip was maintained below 50% in order to observe the cell morphology clearly. Cells were seeded for 24 h followed by fixation in 4% paraformaldehyde for 10 min and PBS washes (three times for 10 min). The fixed cells were then permeabilized using 0.1% Triton X-100 for 3 min, washed with PBS, and then blocked for 1 h using 5% goat serum (Sigma) in 0.1% Triton X-100. Cells were incubated with anti-vinculin antibody (1 : 50; Sigma) for 40 min followed by the staining of Alexa Fluor 594-conjugated goat

anti-mouse IgG (1 : 200; Life Technologies). The actin cytoskeleton was labeled with Alexa Fluor 488 Phalloidin (1 : 150; Invitrogen) for 20 min. Nuclear staining was performed using DAPI. Lastly, the cells were mounted on glass slides and images were obtained using a Leica (Wetzlar, Germany) SP5 confocal microscope.

Quantification of focal adhesions and filopodial metrics

The total number of focal adhesions per cell was quantified manually. The software IMAGEJ (Bethesda, Maryland, US) was used to facilitate the identification of the focal adhesions associated with stress fibers. The 'threshold' function of IMAGEJ was used in vinculin-stained images, and focal adhesions were converted into 'ImageJ selections'. The selections were overlapped into phalloidin-stained images facilitating the identification of the vinculin-positive areas that are associated with actin stress fibers. The total number of filopodia per cell and filopodia length was quantified using the FIJI plug-in FiloQuant [29]. The cell perimeter was quantified manually using IMAGEJ, and the filopodia density (ratio between the number of filopodia and the total cell perimeter in each image) was calculated.

Wound-healing assay

Cells were cultured to confluence on 6-well plates for 24 h. The wells were scratched with a 200- μL pipette tip, washed once with culture media, and incubated with DMEM containing 0.5% FBS. Four scratched areas for each sample were marked and photographed with an inverted microscope immediately or at the indicated time points. Migration was evaluated by measuring the gap area in relation to the area value of the initial scratch. IMAGEJ software was used to measure the relative gap area.

Transwell migration assay

The migration assay was carried out using Transwell chamber with 8- μm pore size polycarbonate membrane (Falcon Cell Culture Insert; Becton-Dickinson, Franklin Lakes, NJ, US). Cells were trypsinized, resuspended in serum-free DMEM, and 8×10^4 cells were seeded in the upper chamber. The chamber was placed in a 24-well plate containing DMEM with 10% FBS. Cells were incubated for 16 h at 37 °C. After the incubation period, the filters were fixed with 4% formaldehyde for 20 min and cells located in the lower filter were stained with 0.1% crystal violet for 20 min. Nonmigrant cells on the upper side of the filter were detached with the use of a cotton swab. The migrated cells were counted microscopically and five fields were counted for each assay.

Statistical analysis

All data are shown as means \pm SD unless otherwise indicated. All statistical significance was determined using a Student two-tailed *t*-test, and *P* values less than 0.05 were considered statistically significant. GRAPHPAD PRISM (GraphPad Software, San Diego, CA, US) was used to plot the mean, SD, and SEM of the data.

Results

The role of RNF8 in the expression of Rho GTPases

To investigate whether proteins involved in cell motility and cytoskeletal dynamics such as GTPases would be regulated by RNF8, we generated RNF8 knockdown cells by infecting BT549 and MDA-MB-231 TNBC cells with lentiviruses containing shRNA targeting luciferase (shLUC) or RNF8 (shRNF8). Immunoblot analysis was performed to determine the effects of RNF8 knockdown in the protein levels of the three most studied Rho GTPases: RAC1, RHOA, and CDC42. Although we observed no changes in the protein level of RAC1 in BT549 cells as previously reported (Fig. 1A) [22], obvious downregulation of RHOA and CDC42 was found upon RNF8 knockdown (Fig. 1B). We used β -actin as a loading control since previous studies showed that depleting RHOA and CDC42 expression does not affect β -actin expression [30,31]. In addition, we used GAPDH as another loading control to confirm that our protein loading among different experimental groups is comparable. Similar results were also obtained in MDA-MB-231 cells when RNF8 expression was inhibited (Fig. 1C,D). Besides TNBC cells, protein expression of RHOA and CDC42 was decreased in HeLa cells, a cervical cancer cell model (Fig. 1E). These results suggest that RNF8 is involved in the regulation of the protein expression of RHOA and CDC42 and the effect is not limited to TNBC cells.

RNF8 regulates the formation of focal adhesions, stress fibers, and filopodia

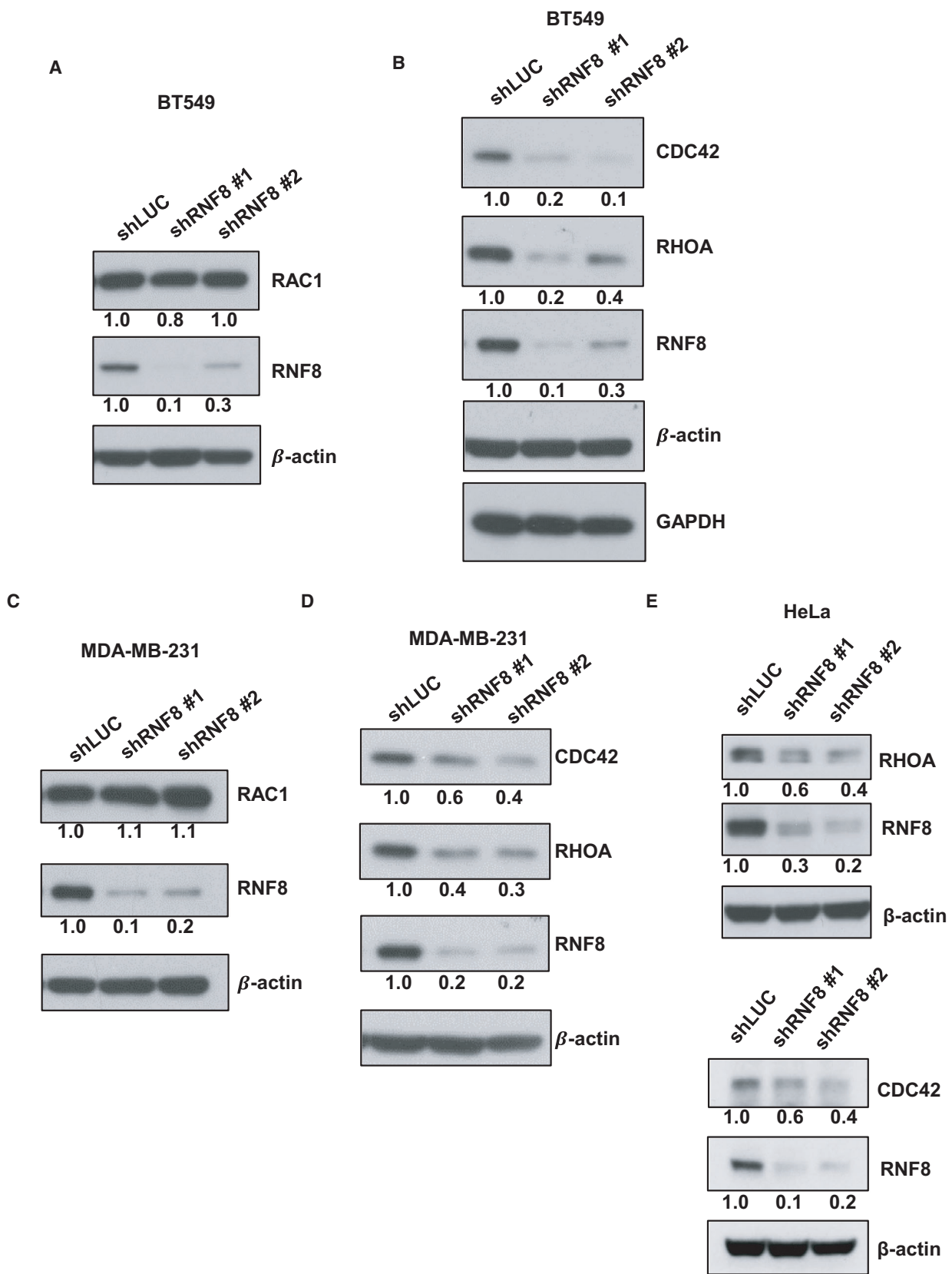
As RHOA and CDC42 are known to be important modulators of actin cytoskeleton, we next investigated

whether RNF8 is also involved in the modulation of the higher cytoskeletal structures composed of actin. We firstly examined the impacts of RNF8 knockdown on the formation of stress fibers, which is known to be mainly regulated by RHOA. BT549 cells with RNF8 or LUC knockdown were seeded on coverslips in low density and stained with Alexa Fluor 488-conjugated Phalloidin to detect F-actin and with anti-vinculin primary antibody and Alexa Fluor 594-conjugated secondary antibody to detect focal adhesions (Fig. 2A). Confocal microscopy analysis revealed a significant decrease in the number of vinculin-positive focal adhesions in RNF8 knockdown cells compared with the control (Fig. 2B). The number of focal adhesions associated with stress fibers was also decreased upon RNF8 knockdown (Fig. 2C). These results suggest that RNF8 may be an upstream regulator of focal adhesion and stress fiber formation, possibly through its modulation over RHOA expression. We next examined whether RNF8 knockdown would also impair the formation of filopodia, the actin-rich protrusion regulated by CDC42 [4]. Control and RNF8 knockdown BT549 cells were seeded on coverslips and stained with Alexa Fluor 488-conjugated Phalloidin to detect filopodial protrusions in the cell membrane (Fig. 2D). Quantification analysis shows that cells with RNF8 knockdown presented a significantly reduced number of filopodia (Fig. 2E), filopodia length (Fig. 2F), and filopodia density (Fig. 2G). The impact of RNF8 knockdown in filopodia formation may be explained by the reduced CDC42 protein levels observed previously (Fig. 1B,D). These results indicate that the depletion of RNF8 not only downregulates CDC42 protein levels but also impairs the CDC42-regulated cytoskeletal structure filopodia. As actin protrusions are the initial step in the cell migration cycle [8], the impairment of filopodia formation upon RNF8 depletion suggests a potential new role of RNF8 in TNBC metastasis.

RNF8 regulates gene transcription but not protein degradation of RHOA and CDC42

To understand the underlying mechanism by which RNF8 modulates the expression of Rho GTPases, we firstly examined the proteasomal degradation system

Fig. 1. RNF8 modulates the protein expression of RHOA and CDC42 but not RAC1 in TNBC cells. (A–D) Immunoblot analysis of the protein expression levels of Rho GTPases in BT549 (A, B) and MDA-MB-231 (C, D) cells with luciferase (shLUC) or RNF8 (shRNF8) knockdown. (E) Immunoblot analysis of the protein expression levels of RHOA and CDC42 in HeLa cells with luciferase (shLUC) or RNF8 (shRNF8) knockdown. The protein expression levels of RAC1, RHOA, and CDC42 were examined and quantified against loading control β -actin using IMAGEJ software (*n* = 3).



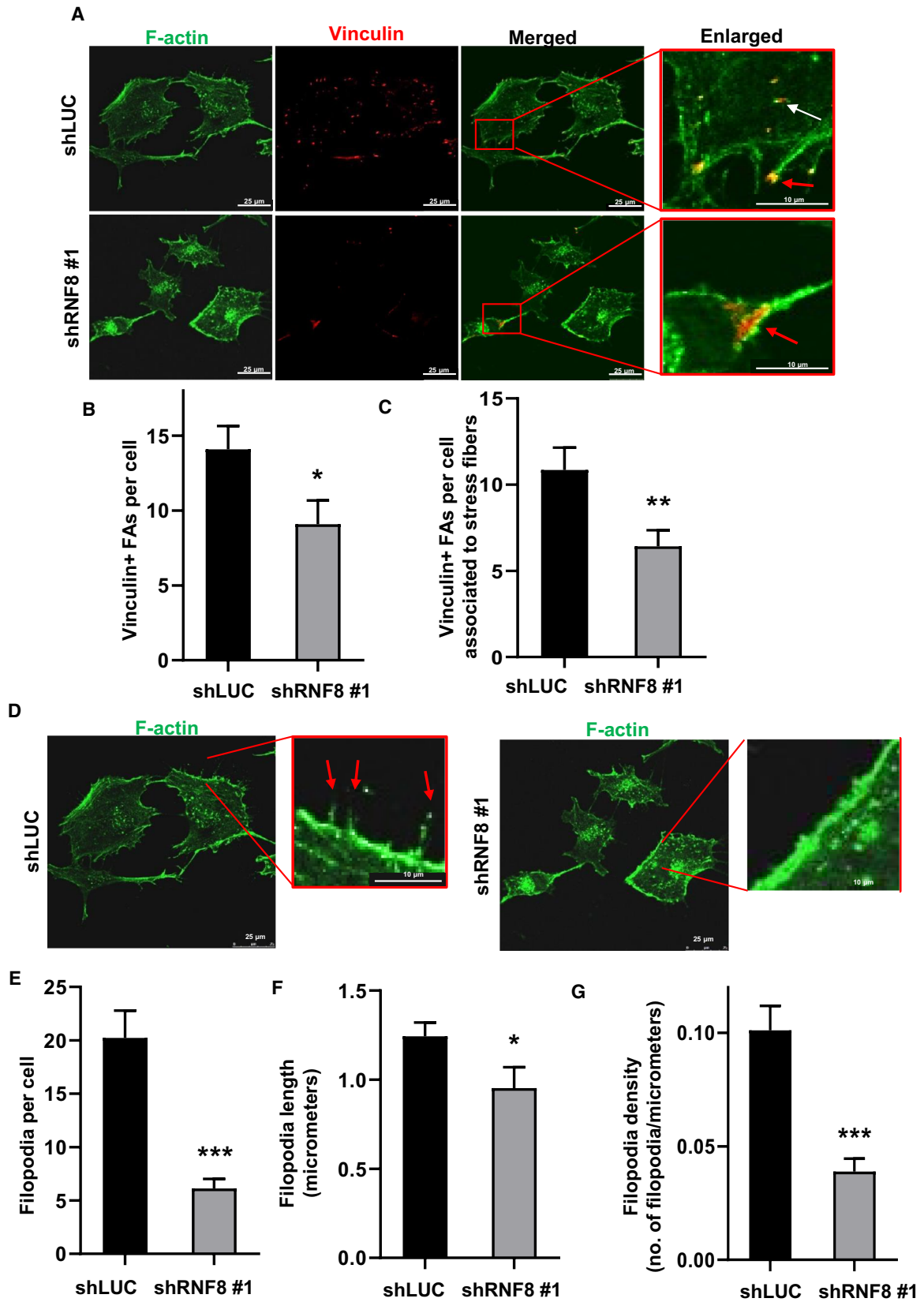


Fig. 2. Knockdown of RNF8 suppresses the formation of focal adhesion and filopodia in BT549 cells. (A) BT549 cells with RNF8 or LUC knockdown were stained with Alexa Fluor 488 Phalloidin (green) and anti-vinculin antibody (red) followed by confocal immunofluorescence analysis. Focal adhesion associated with stress fibers is indicated by a red arrow, while the ones not associated with stress fibers are indicated by white arrow. (B, C) Quantification analysis was performed to determine the total number of focal adhesions (B) and the total number of focal adhesions associated with stress fiber (C) per cell in each cell line ($n = 26$). (D) BT549 cells with RNF8 or LUC knockdown were stained with Alexa Fluor 488 Phalloidin to detect the formation of filopodia. (E, F) Quantification analyses of the number of filopodia per cell (E) and filopodia length (in micrometers) (F) in each cell line were performed using IMAGE J plug-in FiloQuant ($n = 26$). (G) Filopodia density (ratio between the number of filopodia and the perimeter of each cell. The perimeters (in micrometers) of the cells were obtained manually using IMAGEJ.) ($n = 26$). Scale bar = 25 μm (insets = 10 μm) Results represent the means \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

as RNF8 functions as a ubiquitin E3 ligase. BT549 cells with RNF8 or LUC knockdown were treated with proteasome inhibitor MG-132. TWIST protein stability was known to be regulated by RNF8 [22]. Our data demonstrated that MG-132 was able to revert the protein expression of TWIST but not RHOA nor CDC42 in the RNF8 knockdown cells (Fig. 3A). These results suggest that RNF8 does not stabilize RHOA and CDC42 protein *via* ubiquitination-mediated protein degradation. Overexpression of either WT or the Δ RING mutant (an enzymatically inactive mutant) of RNF8 rescued CDC42 expression in RNF8-knockdown cells (Fig. 3B), suggesting that the regulation of RNF8 in CDC42 does not depend on enzymatic activity of RNF8. As RNF8 has been shown to mediate histone H3 modification and promotes the transcription of the downstream genes [28], we next investigated whether RNF8 modulates the expression of these Rho GTPases at transcriptional level. Real-time PCR analysis demonstrated that the mRNA levels of both *RHOA* and *CDC42* were down-regulated in BT549 (Fig. 3C) and MDA-MB-231 (Fig. 3D) cells upon RNF8 knockdown, suggesting that RNF8 exerts transcriptional regulation on these Rho GTPases.

RNF8 regulates cell motility of TNBC cells

Cell migration is initiated by the protrusion of the leading edge composed of filopodia [10,32], while stress fibers associated with focal adhesion function in adhesion and contraction to facilitate the migration process [8]. As we observed RNF8 knockdown led to the reduced protein and mRNA levels of Rho GTPases and subsequent cytoskeletal changes, we next asked whether these changes in molecular levels can be reflected in the decreased cell motility. We therefore performed a wound-healing assay using BT549 cells to evaluate the effect of RNF8 knockdown on cell migration (Fig. 4A). The line between unreached migrating

cells was recorded at 0, 12, 24, and 36 h postscratching. As shown in Fig. 4A, the cell migration was significantly inhibited in shRNF8 cells when compared to the control (Fig. 4B). Quantification of wound closure showed that the difference in migration ability in the control and knockdown cells emerged at 12 h after scratching: We observed that the wound area in control cells accounted for $62 \pm 4.4\%$ of the original wound area, while the knockdown cells infected with two different shRNA both demonstrated slower migration, with $73 \pm 4.7\%$ and $78 \pm 5.4\%$ wound areas, respectively. The effect became more pronounced at 36 h postscratching: The percentage of wound area in control cells was $36 \pm 4.8\%$, while the RNF8 knockdown cells presented a wound area of $51 \pm 9.2\%$ and $66 \pm 2.7\%$. In line with the observation, the transwell migration assay showed that RNF8 knockdown decreases the migration ability of BT549 cells (Fig. 4C). These results demonstrate that RNF8 depletion attenuates the cell motility of TNBC cells in a time-dependent manner, implying a regulatory role of RNF8 in cell migration.

Discussion

In this study, we identified RNF8 as a novel regulator of Rho GTPases RHOA and CDC42 in TNBC. Our data show that RNF8 knockdown attenuates the protein and mRNA expression of RHOA and CDC42, resulting in the impaired focal adhesions, stress fibers, and filopodia formation. The depletion of RNF8 also decreases the number of focal adhesions associated with stress fibers. Importantly, we also observed impaired cell motility in TNBC cells upon RNF8 knockdown, potentially as a result of these cytoskeletal changes. Our study unveils a new role of RNF8 in cell migration by modulating GTPases RHOA and CDC42 in TNBC. Further studies using *in vivo* models are needed to substantiate the role of RNF8-mediated metastasis *via* RHOA and CDC42 in TNBC.

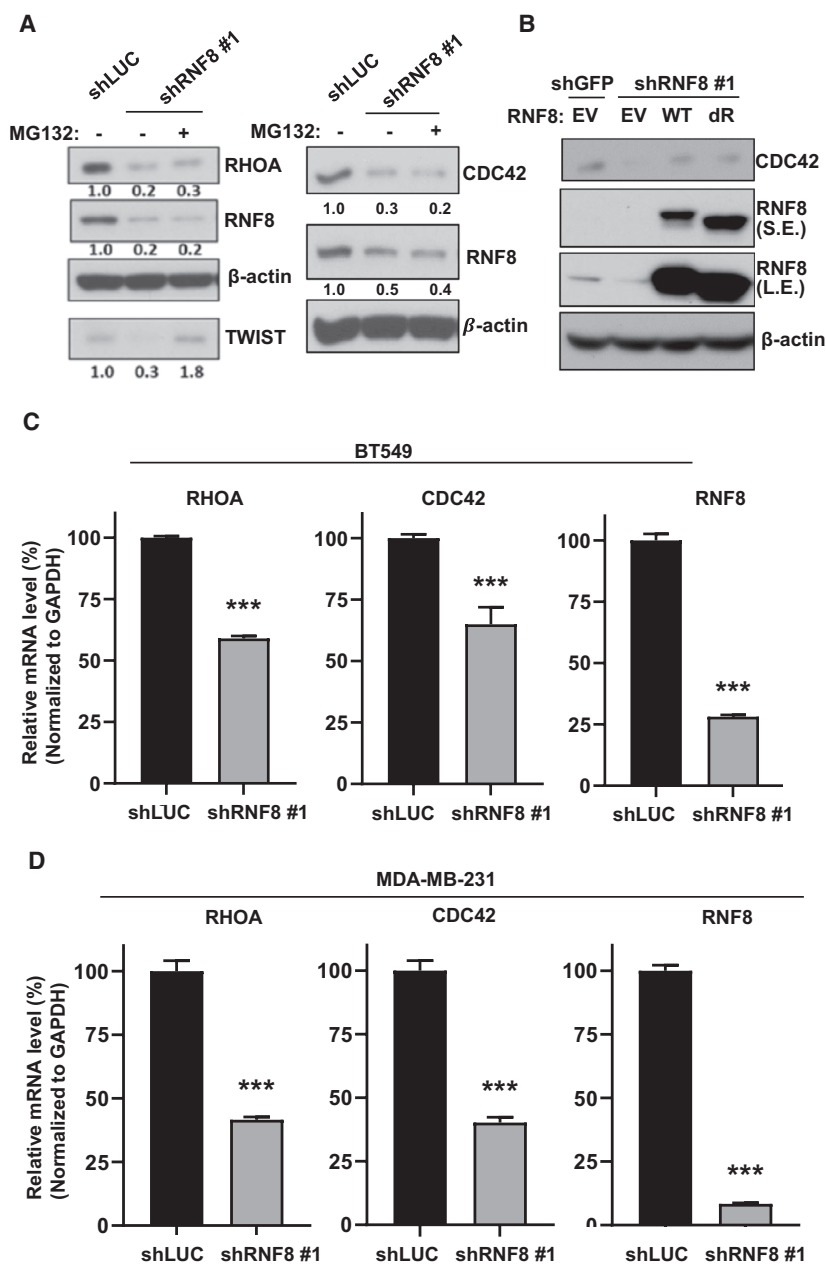


Fig. 3. RNF8 regulates gene transcription but not protein degradation of RHOA and CDC42. (A) BT549 cells with RNF8 or LUC knockdown were treated with vehicle or MG132 (20 μ M for 6 h) followed by immunoblot analysis. The protein expression levels of RHOA and TWIST were quantified against loading control β -actin using IMAGEJ software. (B) Immunoblot analysis of the CDC42 protein expression in RNF8-knockdown BT549 cells with overexpression of vector control, WT, and the Δ RING (Δ R) mutant of RNF8. (C, D) The mRNA expression levels of RHOA, CDC42, and RNF8 in BT549 (C) and MDA-MB-231 (D) cells with RNF8 or LUC knockdown were determined by real-time PCR analysis. Results represent the means \pm SD. *** P < 0.001.

We originally hypothesized that RNF8 could regulate Rho GTPases by K63-linked ubiquitination and protein stabilization [22]. However, the proteasome inhibitor MG132 failed in rescuing the protein levels of RHOA in RNF8 knockdown cells. Our experimental approach using the MG132 inhibitor indicates that RNF8 does not protect RHOA from proteasomal degradation, but it does not rule out the possibility that RNF8 mediates ubiquitination and changes the protein stability of molecules involved in the transcriptional control of these Rho GTPases. This scenario is reported by Xia *et al.*, whose study demonstrated that

RNF8-mediated ubiquitination of histone H3 promotes proteasome-dependent degradation of H3 resulted in nucleosome disassembly. The degradation of H3 ultimately led to the transcription of genes involved in glycolysis and tumorigenesis in glioblastoma [28]. Therefore, one potential mechanism by which RNF8 regulates RHOA and CDC42 in TNBC is through its control over the stability of histone H3 and the associated gene transcription. Further studies examining H3 stability and its effects on cell migration and the expression of RHOA and CDC42 in TNBC cells with RNF8 knockdown are needed to address

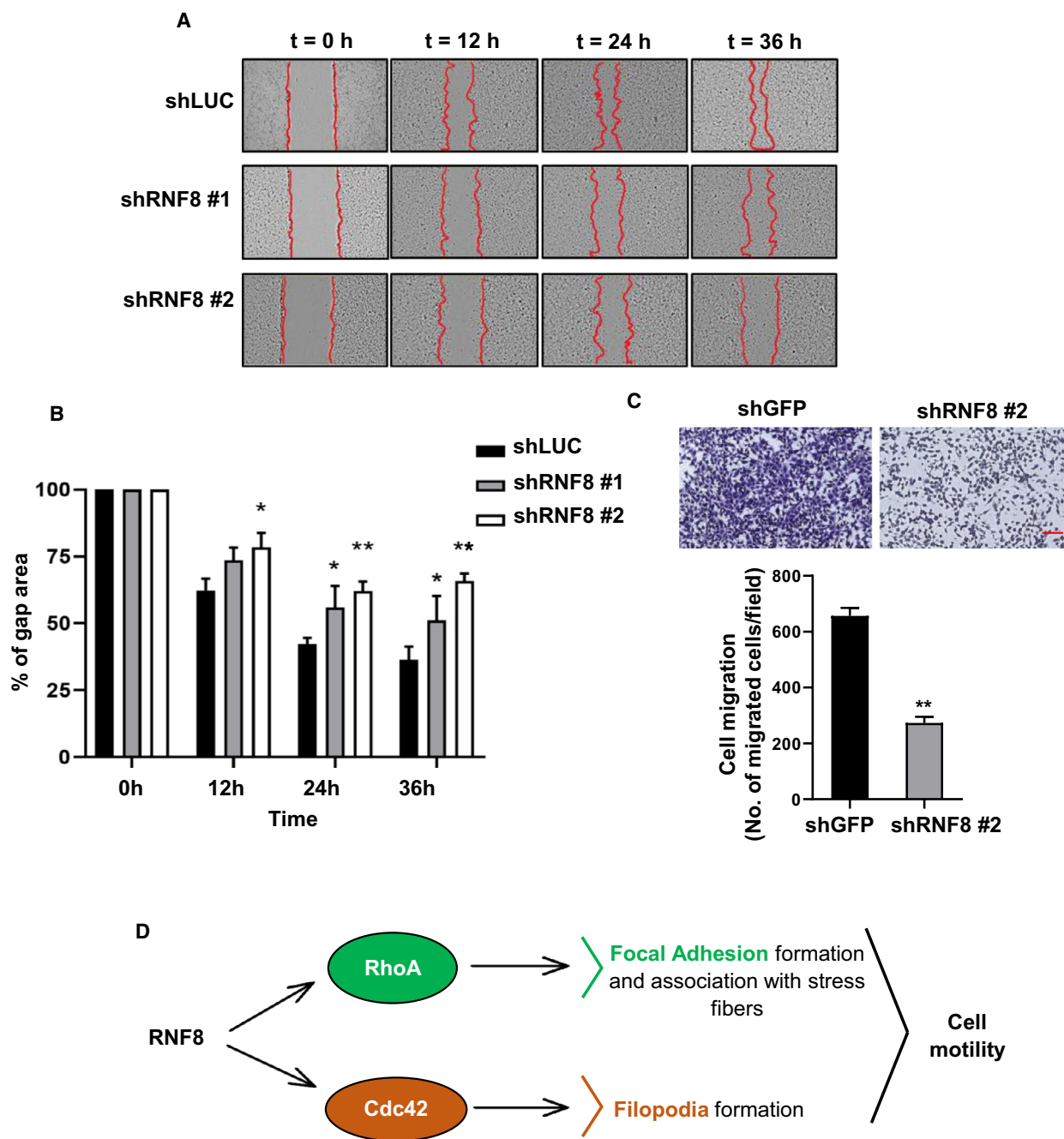


Fig. 4. Knockdown of RNF8 decreases the motility of BT549 cells. (A) Representative images of wound-healing assay in BT549 cells with control (shLUC) or RNF8-knockdown (shRNF8). (B) Cell motility was quantified by measuring the wound area at indicated time points in relation to the area of the initial scratch. Results represent the means \pm SD ($n = 4$). (C) The transwell migration assay in BT549 cells with control (shLUC) or RNF8-knockdown (shRNF8). Results represent the means \pm SEM ($n = 3$). (D) A working model depicting our findings that RNF8 regulates cytoskeletal dynamics and enhances cell motility in TNBC cells *via* its modulation over RHOA and CDC42. * $P < 0.05$; ** $P < 0.01$.

this hypothesis. In addition, it is also important to highlight the role of microRNAs (miRNAs) in the regulation of the mRNA levels of Rho GTPases. These small short noncoding RNA molecules can silence

target genes by either suppressing translation or degrading mRNA, and multiple miRNAs have been reported to target Rho GTPases [33,34]. Future investigations would be needed to examine whether RNF8

regulates mRNA levels of *RHOA* and *CDC42* through miRNAs.

It is worth noting that the impact of RNF8 depletion in RHOA and CDC42 protein levels was more pronounced than the changes observed in mRNA levels. Therefore, RNF8 may still promote the protein stability of RHOA and CDC42 in addition to its control over their gene transcription. RHOA proteolysis is reported to be regulated by serine proteases, calpain, and caspases [35]. Autophagy also contributes to RHOA degradation [36]. CDC42 is susceptible to caspase-catalyzed proteolysis [37]. It is possible that RNF8 disrupts any of these proteolytic components resulting in the protein stabilization of Rho GTPases. More studies are needed to investigate the role of RNF8 in the regulation of these mechanisms.

Although the protein levels of RAC1 do not change upon RNF8 knockdown, RAC1 may still be involved in RNF8-mediated cell migration as our previous study demonstrated that RNF8 knockdown decreased RAC1 activity in BT549 cells without affecting its protein expression [22]. We also found in the same study that RNF8 promotes the activation of the transcription factor TWIST. This suggests the possibility that TWIST might be involved in the RNF8-induced RAC1 activation. It is reported that TWIST cooperates with BMI1 and suppresses the miRNA let-7i, which results in the upregulation of the GEFs NEDD9 and DOCK3, leading to RAC1 activation [38]. Beyond this mechanism, there are multiple GEFs and GAPs involved in the regulation of RAC1, CDC42, and RHOA activity. Further studies are required to determine whether these regulatory proteins are regulated by RNF8.

Several E3 ligases have been previously reported to promote ubiquitination and degradation of RHOA and CDC42. E3 ligases such as CULLIN3 [39,40], FBXL19 [41], FBXW7 [42], and SMURF1 [43,44] are reported to ubiquitinate and promote proteasomal degradation of RHOA. Degradation of CDC42 is reported to be mediated by ligases XIAP [45] and FBXL19 [46]. Unlike the previously reported downregulation caused by E3 ubiquitin ligases, our study showed that RNF8 promotes the upregulation of Rho GTPase protein levels, expanding the knowledge on the diverse impact of E3 ubiquitin ligases in Rho GTPases.

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Author contributions

BDC performed most of the experiments and participated in manuscript writing; YJC wrote the manuscript and performed the rescue experiment; JH contributed to data analysis and performed experiments for revision; and CHC conceived the project, provided overall guidance, and revised the manuscript.

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