


ORIGINAL ARTICLE

Notch-1 signaling promotes reattachment of suspended cancer cells by cdc42-dependent microtentacles formation

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Abstract

Circulating tumor cells (CTCs) are associated with a higher risk of metastasis in tumor patients. The adhesion and arrest of CTCs at a secondary site is an essential prerequisite for the occurrence of tumor metastasis. CTC reattachment has shown to be dependent on microtentacle (McTN) formation in vivo. However, the specific molecular mechanism of McTN formation in suspended cancer cells remains largely unclear. Here, we demonstrated that the activation of Notch-1 signaling triggers McTN formation to facilitate cell reattachment in suspended cell culture conditions. Moreover, molecular mechanistic studies revealed that McTN formation is governed by the balance between microtubule-driven outgrowth and actomyosin-driven cell contractility. The activation of Notch-1 downregulates the acetylation level of microtubules via the Cdc42/HDAC6 pathway, which contributes to microtubule polymerization. Simultaneously, Notch-1 signaling-induced Cdc42 activation also reduced phosphorylation of myosin regulatory light chain, leading to cell contractility attenuation. Altogether, these results defined a novel mechanism by which Notch-1 signaling disturbs the balance between the expansion of microtubules and contraction of the cortical actin, which promotes McTN formation and cell reattachment. Our findings provide a new perspective on the effective therapeutic target to prevent CTC reattachment.

KEYWORDS

cell reattachment, cytoskeleton, microtentacles, Notch-1 signaling, suspended tumor cells

1 | INTRODUCTION

Cancer metastasis is the major cause of most cancer-associated mortality. Metastasis is a complex multistage process in which tumor cells invade the primary tumor tissue, enter the lymphatics or the bloodstream, survive and arrest in the circulation, eventually

extravasate into a tissue, and grow at new sites.¹ To form distant metastases, circulating tumor cells (CTCs) must attach at secondary sites where they are arrested through adhesion to endothelial cells.^{2,3} Once detach from the extracellular matrix and enter into the circulatory system, the CTCs undergo both morphological and structural changes to adapt to the new microenvironment.⁴ Recent studies

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showed that CTCs extend long and dynamic membrane protrusions termed microtentacles (McTNs).⁵⁻⁷ Emerging evidence indicates that McTNs could enhance cell-cell aggregation and facilitate tumor cell reattachment to the endothelial monolayer or extracellular matrix.^{4,8} It is reported that cells forming McTNs are more efficiently retained in the microvasculature in the lungs of mice.⁹ Although numerous factors are revealed to induce McTNs formation and lead to cancer malignant progression and metastasis enhancement, the underlying mechanisms of McTNs formation still remain largely unclear.

McTNs, a novel cellular substructure in suspended culture conditions, are characterized by cell membrane microtubule-based protrusions that extend 10–100 μm from the cell body and regulate by an actin-microtubule balance.⁷ Mammary epithelial cells perform a delicate balance between 2 opposing cytoskeletal forces to stabilize cell shape.⁵ Conversely, in suspended cancer cells, the stabilized microtubules could overcome the restrictive forces of the actin cortex.^{10,11} This suggests that the deformable cytoskeleton contributes to McTN formation. Cdc42, one of the small cytoskeletal Rho-GTPases, plays a primary role in cell plasticity and cytoskeletal dynamic, leading to changes in cell morphology and polarity.¹²⁻¹⁴ Cdc42 acts as molecular switches by alternating between their active GTP-bound form and inactive GDP-bound form in cells.¹⁵ Some data have supported that aberrant Cdc42 activity is implicated in many processes of tumor metastasis.¹⁶ Especially, Cdc42 could facilitate tumor cell adhesion and spread to endothelial cells and is unique in strongly promoting transendothelial migration as well as early colonization in the lung *in vivo*.¹⁷ Although a great deal of work has been done to study the mechanisms involved in Cdc42-mediated cell motility, how Cdc42 triggers cytoskeleton remodeling to induce McTN formation is yet to fully elucidated. Given that the change in Cdc42 activity is modulated by signaling through cell surface receptors, recent progress has demonstrated that Cdc42 is not only a regulator of cellular mechanical behaviors but also a transducer of various signals.¹⁵

The Notch signaling pathway is an evolutionarily conserved signaling pathway that has been shown to regulate many cellular processes including cell proliferation, differentiation, apoptosis, and survival.¹⁸ In mammalian cells, Notch signaling pathways include 4 transmembrane receptors (Notch-1 to Notch-4) and 5 ligands (Delta-like 1, 3, 4, Jagged 1, 2).^{19,20} The Notch-1-mediated signaling pathway plays a crucial role in malignant features of cancer cells and cell fate decisions.²¹⁻²⁴ Recently, it was reported that Notch-1 activation and epithelial-mesenchymal transition (EMT) are coupled to promote squamous cell carcinoma initiation, which suggested that Notch-1 signaling could regulate cell morphology, deformation, and cell motility.²⁵⁻²⁷ More recent studies have also shown that it is increasingly clear that Notch signaling also has roles in the regulation of synaptic plasticity and neuronal migration, suggesting closely related functions of Notch-1 and cytoskeleton reorganization.²⁸⁻³⁰ Importantly, earlier research also discovered that cancer cells that exhibit mesenchymal and stem cell phenotypes trigger McTN formation and reattachment.^{31,32} In light of these previous findings, we questioned how Notch-1 signaling participates in cell reattachment

and the precise molecular mechanisms through which Notch-1 signaling regulates McTNs formation.

Here, we aimed to define the functional role of Notch-1 signaling in cancer cell reattachment. In this study, we demonstrated that Notch-1 activation-triggered signaling increased the outward force, driven by microtubules, as well as suppressed the inward contractile force, driven by cell contractility of the actin cortex. Mechanistically, Notch-1-induced Cdc42 activation could downregulate HDAC6 to increase the acetylation of α -tubulin, and resulted in the outward expansion of microtubules. Activated Cdc42 also decreased the phosphorylation of myosin light chain (MLC) to inhibit actomyosin contractility. Collectively, Notch-1-modulated cytoskeleton reorganization contributes to McTN formation and reattachment of cancer cells in suspended culture conditions. These findings suggested an important role for Notch-1 signaling in cancer cell malignant metastasis and identified the Notch-1/Cdc42 signaling axis as a potential target for cancer metastasis therapy.

2 | MATERIALS AND METHODS

2.1 | Cell culture

MDA-MB-231 and MCF-7 cells were obtained from the American Type Culture Collection. MDA-MB-231 cells were maintained at 37°C in Leibovitz's L-15 (Thermo Fisher Scientific) culture medium supplemented with 10% FBS and 1% penicillin-streptomycin. MCF-7 cells were maintained at 37°C in DMEM (Thermo Fisher Scientific) supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were passaged every 5 d and were harvested using 0.25% trypsin-EDTA (Thermo Fisher Scientific).

2.2 | Time-lapse microscopy

Suspended cultured cells was performed by time-lapse microscopy. The plasmid EGFP-tubulin was transiently transfected into cells for microtubules dynamics evaluation. Confocal laser scanning microscopy (FV1000) was used for imaging. For recording cell behavior, images were continuously captured for 2 min.

2.3 | Cell-reattachment assay

Real-time cell reattachment was performed as described previously⁶ and measured using the Electric Cell-Substrate Impedance Sensing (ECIS) device (Applied Biophysics) in accordance with the manufacturer's protocol. Briefly, 20 000 cells per well were added to electrode-containing microtiter plates (8W10E) for reattachment. Reattachment rates was quantitatively recorded as Cell Index (a change in electrical impedance of the current flowing through the electrodes). Impedance was recorded for 2 h for reattachment. Raw data were exported to Microsoft Excel. Graphs shown are

representative of a single experimental run \pm standard deviation of at least 3 wells.

2.4 | Fluorescence recovery after photobleaching (FRAP)

FRAP analysis was performed as described previously.³³ FRAP was performed on a confocal microscope (ZEIS LSM800). Cells were seeded at low density in a 12-well plate for 24 h and transfected with mCherry/GFP- α -tubulin, and experiments were performed 48 h later. After acquiring prebleached images, a small area within the microtubule in peripheral protrusion was bleached with the 488/561 line of the SIM scanner and recovered until the intensity reached a plateau and normalized to the prebleaching intensity.

2.5 | Fluorescence resonance energy transfer (FRET)

MDA-MB-231 cells transiently expressing YFP/CFP-tagged Cdc42-GTP proteins were grown on glass coverslips. Cells were imaged with an inverted microscope equipped with a live-cell imaging system. FRET measurements were performed as previously described.³⁴ To visualize CFP and YFP, consecutive images were acquired sequentially through YFP (excitation, 500/20 nm; emission, 535/30 nm), CFP (excitation, 436/10 nm; emission, 470/30 nm), and FRET (excitation, 436/10 nm; emission, 535/30 nm) filter channels. After data acquisition, the average intensities of CFP, YFP and FRET were measured and fluorescence was calculated through the FRET filter set consisting of a FRET component ('corrected' FRET [FRET]). The background images were subtracted from raw images prior to carrying out FRET calculations.

2.6 | Metastasis assay

Here, 6–8-wk-old BALB/c nude female mice were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. In vivo lung retention assay was performed as previously reported.^{9,17} Briefly, cells were resuspended at 3×10^6 cells/mL in phosphate-buffered saline. Then, 3×10^5 cells were injected into the tail veins

of mice. The mice were anesthetized with isoflurane gas (integrated within the Lumina Series III imaging). Total photon flux (photons/sec) was calculated and corrected by spectral imaging using Living Image software.

2.7 | Statistical analysis

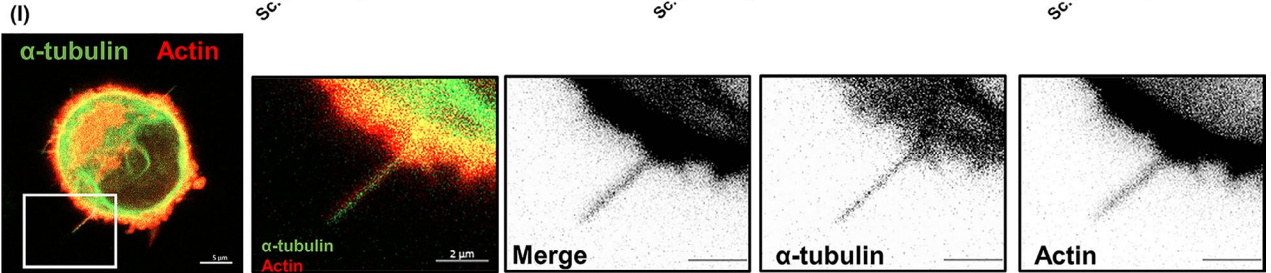
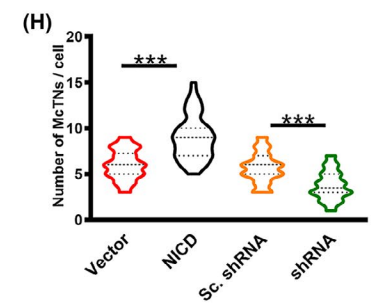
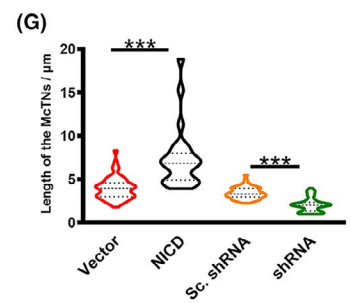
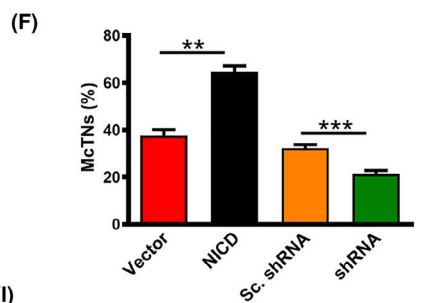
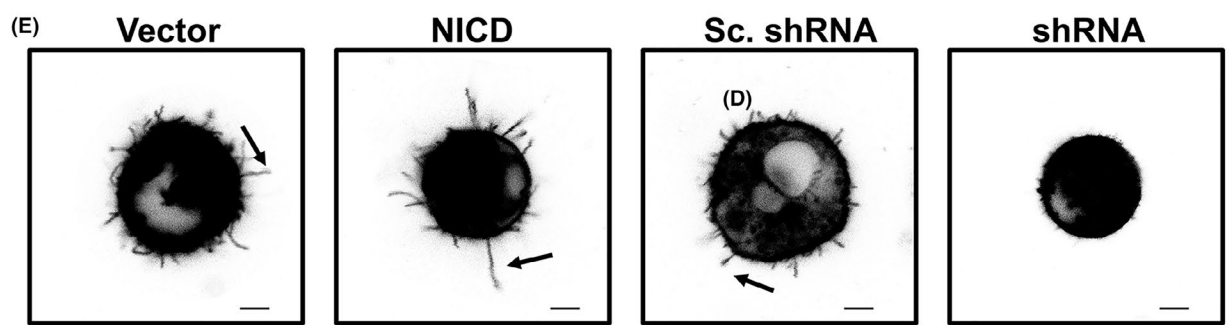
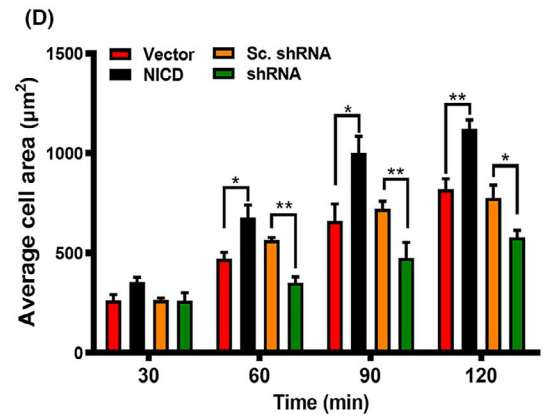
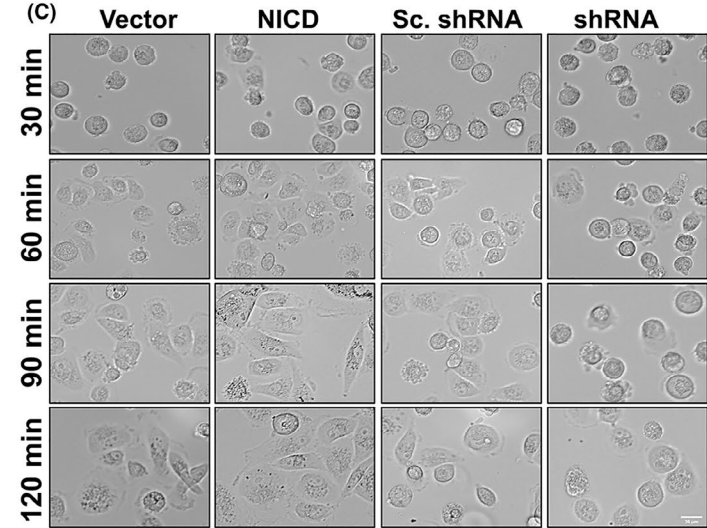
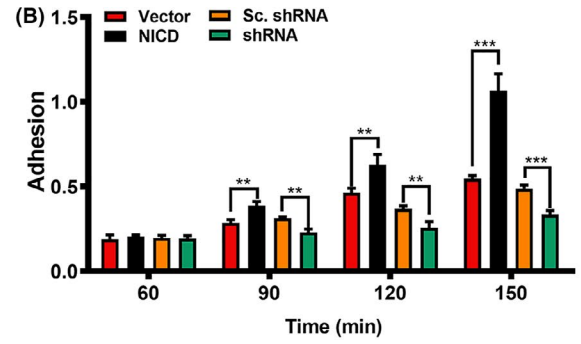
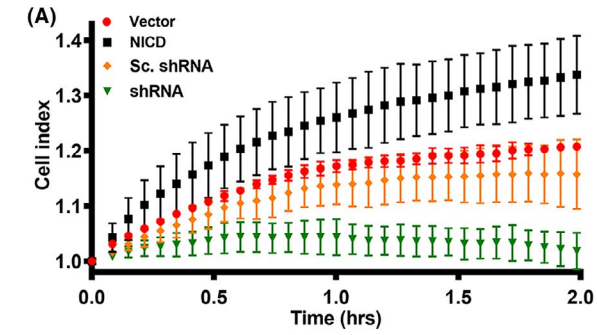
Each experiment was performed at least 3 times. All data were expressed as the mean \pm standard deviation (SD) using GraphPad Prism software, version 8.0. Statistical analyses were performed with Student *t* test or one-way analysis of variance with post hoc multiple comparisons. The significant differences between groups were considered at a minimum value of $P < .05$.

3 | RESULTS

3.1 | Notch-1 signaling increases the reattachment of suspended breast cancer cells

To investigate the potential role of Notch-1 signaling in cancer cell reattachment, we overexpressed NICD using a constitutively active Notch1 intracellular domain in cells and knockdown of Notch-1 by shRNA. The expression of NICD and Notch-1 silencing were confirmed by western blotting (Figures S1A and S1B,C). We found that Notch-1 activation increased cell reattachment of both MDA-MB-231 and MCF-7 cells. (Figures 1A,B and S1F). Additionally, cell spreading areas were attenuated in Notch-1 knockdown cells compared with the control (scrambled Notch-1 shRNA) (Figure 1C,D). As previously reported, suspended breast cancer cells could form McTNs, which aid in tumor cell aggregation and reattachment. To determine whether Notch-1 activation would induce McTNs formation to facilitate cell reattachment, we assessed the role of NICD in McTN formation by counting the number of observable McTNs. As observed in suspended culture conditions, activated Notch-1 cells exhibited numerous long McTNs (Figures 1E and S1D,E). Knockdown of Notch-1 resulted in *c.* 40% reduction in cells with McTNs compared with the control (Figure 1F). We next measured the number and average length of McTNs for each cell. The number and average length showed a significant reduction in Notch-1 knockdown cells (Figure 1F-H). Using immunofluorescence staining, we found that

FIGURE 1 Constitutive Notch-1 activation (NICD) promotes cell reattachment and spreading. A, Real-time cell reattachment was analyzed using Electric Cell-Substrate Impedance Sensing (ECIS). Cell index represents the variation in electrical impedance over time. Vector: empty vector, NICD: Notch-1 intracellular domain, Sc.shRNA: Scrambled shRNA, shRNA: Notch-1 shRNA. B, Adhesion assays were performed in 96-well plates. Cells suspended in medium were incubated at 37°C. Adherent cells were fixed, stained with 0.1% crystal violet. Absorbance was measured at 570 nm. C, Cell spreading areas were assessed by fluorescence microscope. Images were acquired every 30 min for 2 h. Scale bar = 20 μ m. D, Quantification of the cell spreading area was determined by calculating the marked area in 3 randomly selected fields, and the data are presented as the mean \pm SEM. ****** $P < .01$. E, Cells were suspended for 30 min in ultralow-attach plates and stained with CellMask™ DeepRed cell membrane dye. Arrows showed the representative suspended cells images highlighting McTNs. Scale bars = 5 μ m. F, McTNs counts were carried out on suspended cells, with data represented as the mean \pm SD of 3 independent experiments with at least 100 cells scored blindly in each independent experiment. G, H, The numbers and average lengths of McTNs were calculated on suspended cells, ****** $P < .01$. I, Representative confocal images of the cytoskeleton structure of McTNs. Cells were subjected to suspended cell immunofluorescence for α -tubulin (green) and F-actin (red)



McTNs stained positive for both F-actin and α -tubulin in suspended cells (Figure 1I). These results showed that activation of Notch-1 triggered McTN formation to promote cell reattachment.

3.2 | Notch-1 activation promotes McTNs formation by enhancing microtubule polymerization

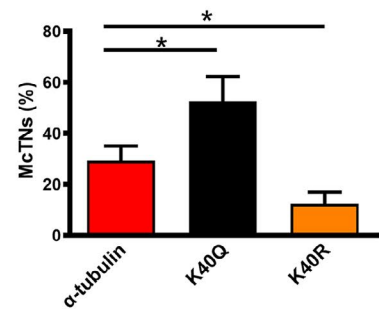
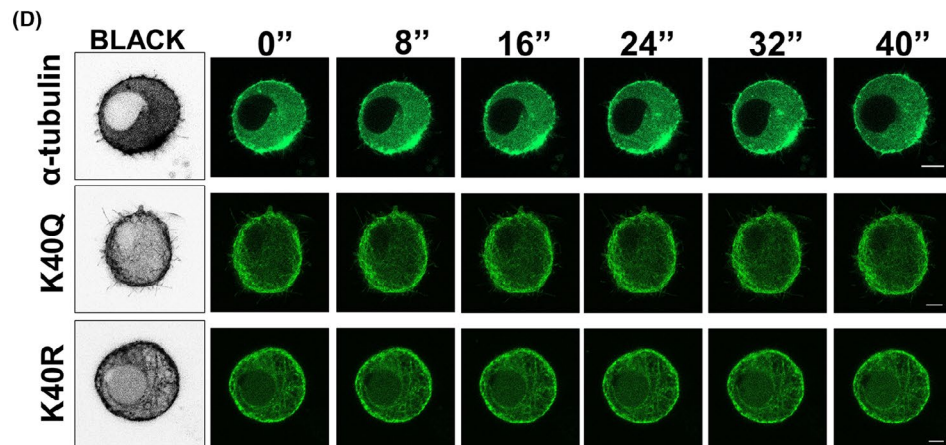
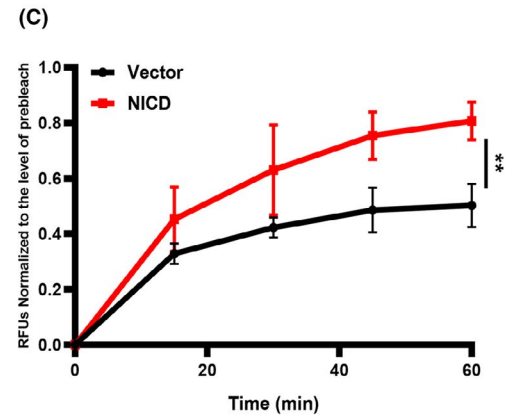
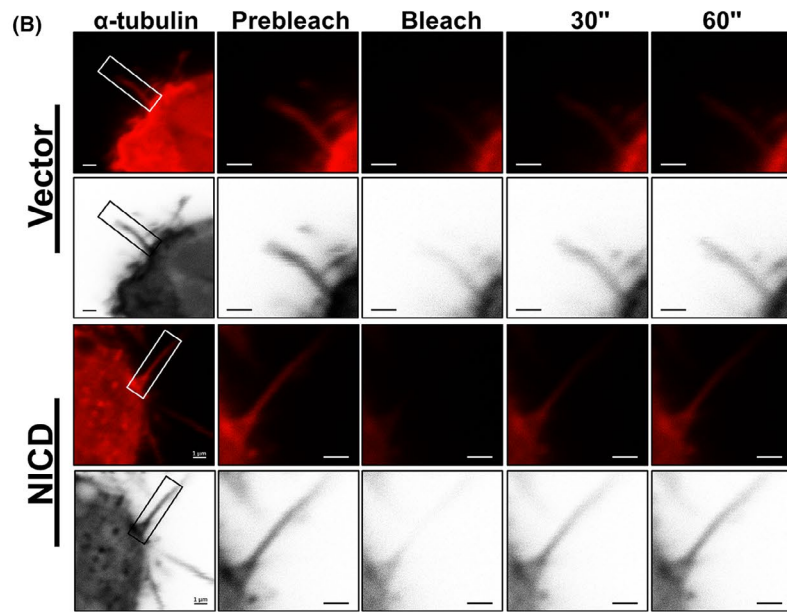
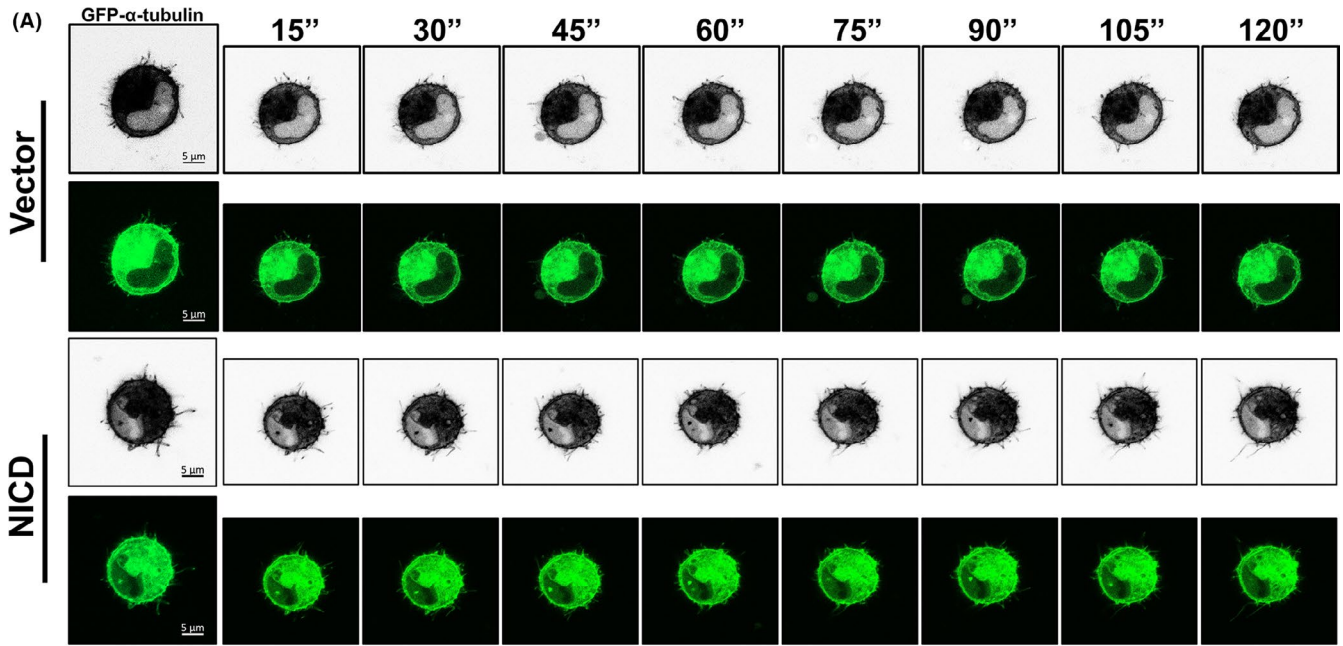
Given the abundance of F-actin and α -tubulin within McTNs structure, we next investigated functional contributions of Notch-1 signaling to McTN morphology. To visualize the effect of Notch-1 activation on microtubule dynamics, cells expressing GFP- α -tubulin were traced with time-lapse imaging (Video S1 and Figure 2A). NICD overexpression (Notch-1 activation) induced microtubule filament outgrowth and displayed long, motile McTNs. Characterization of colchicine-treated cells by fluorescent cytomembrane staining showed that inhibition of microtubule polymerization reduced both the number and average length of McTNs (Figure S2A-C). Convincingly, the results of FRAP analysis also showed that NICD overexpression accelerated the recovery of mCherry- α -tubulin fluorescence signaling after bleaching, suggesting that Notch-1 activation promoted microtubule polymerization (Figure 2B,C). It has been shown that acetylation of α -tubulin, a well known marker of stabilized microtubules, occurs on lysine 40 (K40) by the α -tubulin acetyltransferase 1 and can be reversed by histone deacetylase 6 (HDAC6).^{6,35} As α -tubulin acetylation is associated with tumor metastasis, we then evaluated the levels of acetylation α -tubulin both in MCF-7 (non-metastatic cell line) and MDA-MB-231 (metastatic cell line) cells. It was found that, in suspension culture conditions, endogenous acetylation of α -tubulin in MDA-MB-231 cells was significantly higher compared with that in MCF-7 cells (Figure S5A,B). To explore the effect of Notch-1 on microtubule stability, immunoblotting was performed to detect acetylated α -tubulin. NICD overexpression elevated the acetylation of tubulin and downregulated HDAC6 expression (Figure S2D). Immunofluorescence was performed to visualize the distribution of acetylated α -tubulin and total α -tubulin. Our data showed that acetylated α -tubulin extends along with the McTNs (Figure S3). Next, cells were transfected with different α -tubulin acetylation status mutants and verified by live-cell time-lapse imaging (Figure 2D). Expression of the acetyl-mimetic α -tubulin mutant (K40Q, Hyper-AcMT) induced McTN formation, whereas expression of the acetyl-null α -tubulin mutant (K40R;

Hypo-AcMT) suppressed McTN formation (Figures 2D, S2E, and S5C). Tubulin acetylation was rescued in Notch-1 knockdown cells by transfecting with K40Q mutant (data not shown). Expression of K40Q mutant promoted the McTN formation and cell reattachment (Figure S2E,F). We concluded that the activation of Notch-1 contributed to microtubule polymerization to induce McTN formation.

3.3 | Notch-1 activation increases McTNs formation through attenuating cell contractility

We next examined how the actin cytoskeleton regulates McTN formation. Pharmacological approaches were utilized to manipulated actin cytoskeleton dynamics. McTNs remained observed in control cells, whereas Notch-1 knockdown cells displayed fairly short McTNs (Figure S4A-E). Cytochalasin D or blebbistatin treatment resulted in an increase in both the number and average length of McTNs. Subsequently, immunofluorescence staining was used for more visual observation of the McTN structure (Figure 3A). These results mirrored the fact that actin depolymerization inhibited cellular contractility to promote McTN formation. Cofilin, a ubiquitously expressed actin-severing protein, modulates actin filament tension, and contractility.³⁶ If the decreased phosphorylation of cofilin was responsible for McTN formation, the expression of p-cofilin should be downregulated upon Notch-1 activation. Unexpectedly, there was no significant difference in activated or inactivated Notch-1 cells (data not shown). As previously observed, inhibition of myosin II with blebbistatin promotes McTN formation, cells were transfected with activated or inactivated MLC to examine the effect of myosin II on McTNs formation. Expression of MLC-DD (phosphory-mimetic mutant, active myosin) suppressed McTN formation, whereas expression of MLC-AA (phosphory-null mutant, inactive myosin) was able to increase McTNs (Figures 3B and S5C). Myosin activity was also evaluated through immunoblotting. p-MLC levels decreased approximately 1-fold for overexpression of NICD relative to the control (vector group). Notch-1 knockdown cells maintained a higher level of p-MLC (Figure 3C). We further examined whether Notch-1 activation-induced McTN formation could be affected by MLC (Figure S6). The NICD-overexpressed cells were transfected with activated myosin light chain plasmid (MLC-DD), and it was found that activated myosin suppressed McTN formation, whereas inactive myosin (MLC-AA, phosphory-null mutant) could rescue McTN

FIGURE 2 Notch-1 activation (NICD) increased microtubule polymerization in suspended cells. A, Representative frames were cropped from living cells to show cytoskeletal dynamics of McTNs. Cells were transfected with GFP- α -tubulin and recorded by time-lapse microscopy. Representative frames of 15 s intervals are presented for comparison. See also the Video S1. B, Fluorescence bleaching recovery assay was performed to examine the difference in microtubule polymerization ability in cells. Cell samples were placed in a live-cell bench. The bleached area was selected. Time-lapse photography was performed for 60 s continuously immediately after the fluorescence was bleached using a strong laser. Scale bar = 1 μ m. C, The graph reports the average mobile fraction fluorescence intensity in the bleached zone during recovery. Values represent means \pm SD of 3 independent experiments. D, MDA-MB-231 cells were transiently transfected with GFP-tagged wild-type tubulin (α -tubulin; control), GFP-tagged acetyl-mimetic tubulin mutant (K40Q; HyperAcMT), or GFP-tagged acetyl-null tubulin mutant (K40R; HypoAcMT). Then, time-lapse photography was used to observe the difference in McTNs formation. Representative frames of 8 s intervals are presented for comparison. McTNs counts were carried out on suspended cell, with data represented as the mean \pm SD of 3 independent experiments with 30 cells scored blindly in each independent experiment, Scale bars = 5 μ m



formation in Notch-1 knockdown cells. These observations further confirmed that the activation of Notch-1 could reduce actomyosin contractility to induce McTNs formation.

3.4 | Microtubule and actin filaments undergo coordinated extension and retraction in McTNs formation

Previous data demonstrated that microtubule and actin filaments had opposite regulation role in McTN formation. Here, we sought to further characterize the dynamic interplay of microtubules and actin in McTN formation. To perform live-cell experiments and facilitate simultaneous monitoring of both microtubules and actin filaments by a live-cell imaging (Video S2), we generated a GFP-tubulin and mCherry-LifeAct line that was stably expressing GFP (green)-labeled microtubules and mCherry (red)-labeled actin. It was found that suspended cells displayed strong co-localization of microtubules and actin filaments in McTNs (Figure 4A). The adherent cells showed less co-localization of microtubules and actin filaments, with short filopodial structures decorating and outlining the cell periphery (Figure 4B). Notably, microtubules and actin filaments underwent a tendency for outgrowth in McTNs at a similar rate (Figure 4A). These results underscored the mechanistic differences between filopodia and McTNs.

3.5 | Notch-1 signaling activates Cdc42 to induce McTN formation and facilitate cell reattachment

Cdc42 plays a central role in cell migration and invasion by coordinately regulating cytoskeletal dynamics. It predominantly acts on membrane dynamics by regulating actin polymerization. Many studies have also shown that microtubule dynamics during cell migration depends on the activation of Cdc42-related pathways.¹⁴ Previous data showed that both microtubules and the actin cytoskeleton underwent dynamic remodeling in McTNs, and led us to hypothesize that cells may coordinate cellular contractility and microtubule outgrowth. To decipher the cooperative regulation mechanism, we used a photoactivatable Cdc42 plasmid to examine the effect of Cdc42 on McTN formation (Video S3 and Figure 5A,B). By photoactivating the whole cell and real-time tracking the dynamics of microtubules, we found that activated Cdc42 promoted McTN formation. Cells were transfected with Cdc42-WT (control) or Cdc42-Q61L (constitutively active Cdc42 mutant), or Cdc42-T17N (dominant-negative mutant, inactive Cdc42) to verify this phenomenon. As anticipated, activation of Cdc42 increased both the number and average length of McTNs (Figures S7A and 5C,D). We next explored whether Cdc42 activation regulated cytoskeleton remodeling via its downstream signal molecules. HDAC6, acetyl- α -tubulin, and the p-MLC protein expression levels were further detected by using western blotting assay. It was found that

activation of Cdc42 could elevate α -tubulin acetylation by down-regulating HDAC6 as well as suppressing MLC phosphorylation (Figures S7B-D and 5G). FRAP analysis also showed that Cdc42 activation enhanced the recovery of the mCherry- α -tubulin fluorescence signal after bleaching (Figure 5E,F). To further verify that Cdc42 mediated McTN formation by upregulating acetylation of α -tubulin, cells were co-transfected with the EGFP-Cdc42-Q61L and mCherry-tubulin-K40R. We found that Cdc42 activation-initiated McTN formation was suppressed by an acetyl-null α -tubulin mutant (K40R) (Figure S8A). These results indicated that activation of Cdc42 promotes McTN formation. To further investigate the potential relationship between Notch-1 signaling and Cdc42, FRET imaging was used to visualize the Cdc42 activity in living cells. We found that Notch-1 signaling could induce the activation of Cdc42 (Figure 6A). Western blotting also confirmed that Notch-1 knockdown reduced the expression of Cdc42-GTP (Figure 6B). As McTNs formation depended on Cdc42 activity, it was essential to establish whether Cdc42 activation contributed to cell reattachment. We found that expression of a constitutively active Cdc42 mutant in cells (Cdc42-Q61L) showed a higher reattachment ability compared with Cdc42-T17N mutant cells (Figure 6C,D). We next examined whether acetylated α -tubulin would affect Cdc42-induced cell reattachment, we also used ECIS assay and found that the expression of acetyl-mimetic α -tubulin mutant (K40Q) would rescue the effects of dominant-negative Cdc42 (Cdc42-T17N) on cell reattachment (Figure S8B). Collectively, these data suggested that Notch-1 signaling-induced McTNs formation depends on Cdc42 activation.

3.6 | Notch-1 activation promoted cells retaining in the microvasculatures

In vitro data have demonstrated that Notch-1 activation greatly enhanced the reattachment of suspended cancer cells, we further tested whether Notch-1 activation-induced McTN formation was involved in capillary retention of CTC in vivo. To this end, cancer cells were labeled with DIR and injected into the tail veins of mice, and imaged over a 24 h time course (Figure 7B,C). At 2 h post-injection, 60.2% of NICD-overexpressed cells were retained in the lungs, but only 25.2% for Notch-1 knockdown cells. At 24 h, 23.5% of the control cells (Sc. shRNA) remained trapped in the lungs compared with 12.7% of the Notch-1 knockdown cells, suggesting that Notch-1 activation could promote CTCs retained in capillaries. Given that the more retained cells in capillary could increase the subsequent formation of metastases, the lungs of mice were prepared for H&E staining to observe tumor foci formation (Figure 7D,E). We found that Notch-1 knockdown led to a marked reduction in foci number in the lungs. The foci were then categorized based on size. For all the size categories, we found that the number of foci notably decreased in the Notch-1 knockdown group (data not shown). Taken together, our data confirmed that Notch-1 signaling could affect cancer metastasis.

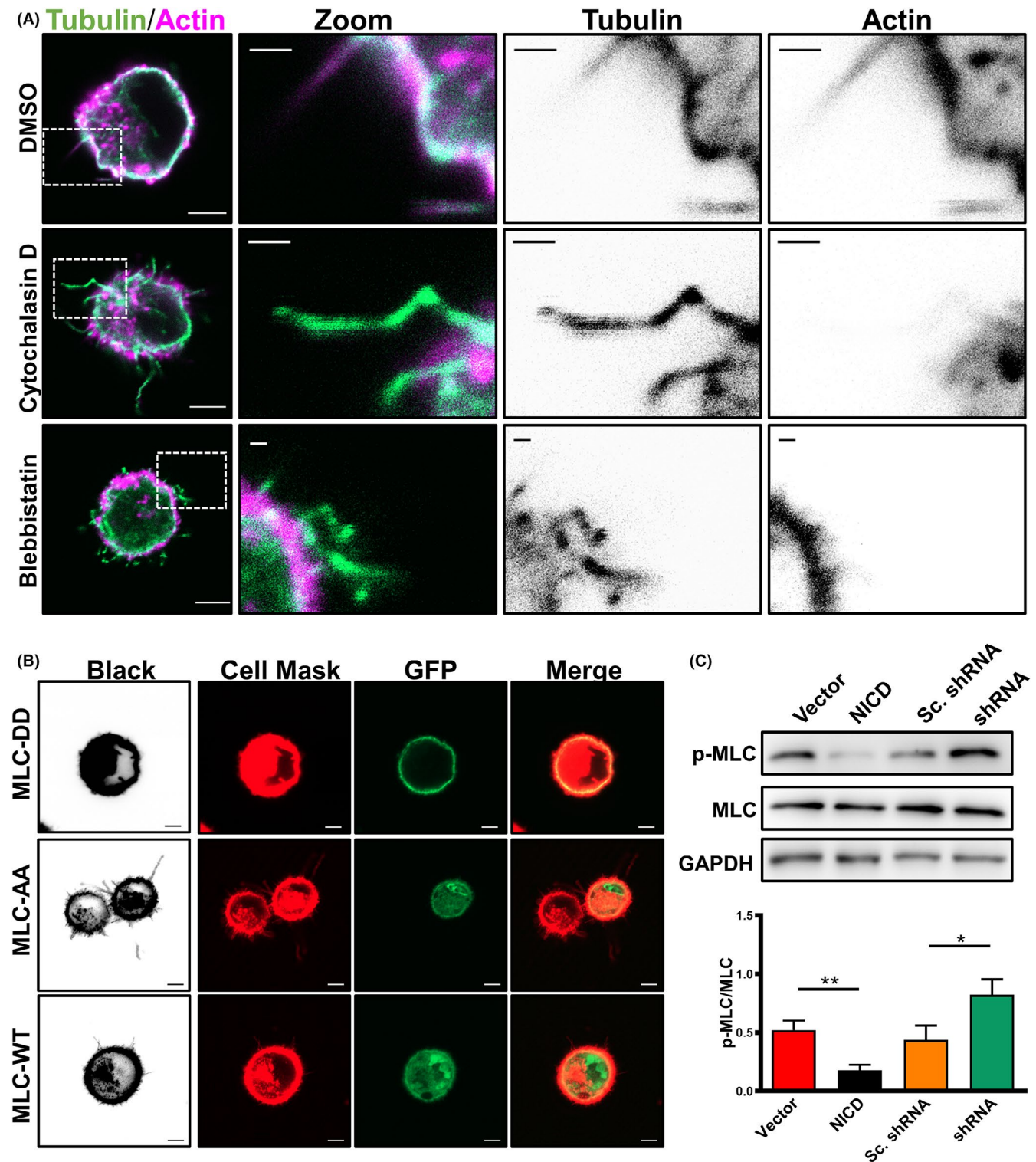


FIGURE 3 Inhibition of actomyosin contraction enhanced MCTNs formation. **A**, Representative confocal images of MCTNs structure. Cells were suspended in low-attachment conditions and treated with Cytochalasin D (10 μ M), or blebbistatin (5 μ M) for 30 min, and cells were fixed and stained for tubulin (green) and F-actin (purple red) to indicate MCTN morphology. White line box represents zoom area. zoom shows protrusions at the cell periphery. Scale bars = 5 μ m (left row). **B**, Live-cell confocal microscopy was used to observe MCTN protrusion. Representative confocal images of MDA-MB-231 cells expressing different MLC mutants: GFP-tagged wild-type MLC (MLC-WT, control), GFP-tagged phosphory-mimetic MLC mutant (MLC-DD, active myosin), or GFP-tagged phosphory-null MLC mutant (MLC-AA, inactive myosin). Scale bar = 5 μ m. **C**, Expression of MLC and p-MLC was detected by western blotting. Histograms reporting the quantitative analysis of p-MLC expression were measured by ImageJ software. Values represent means \pm SEM; n = 3. ** P < .01

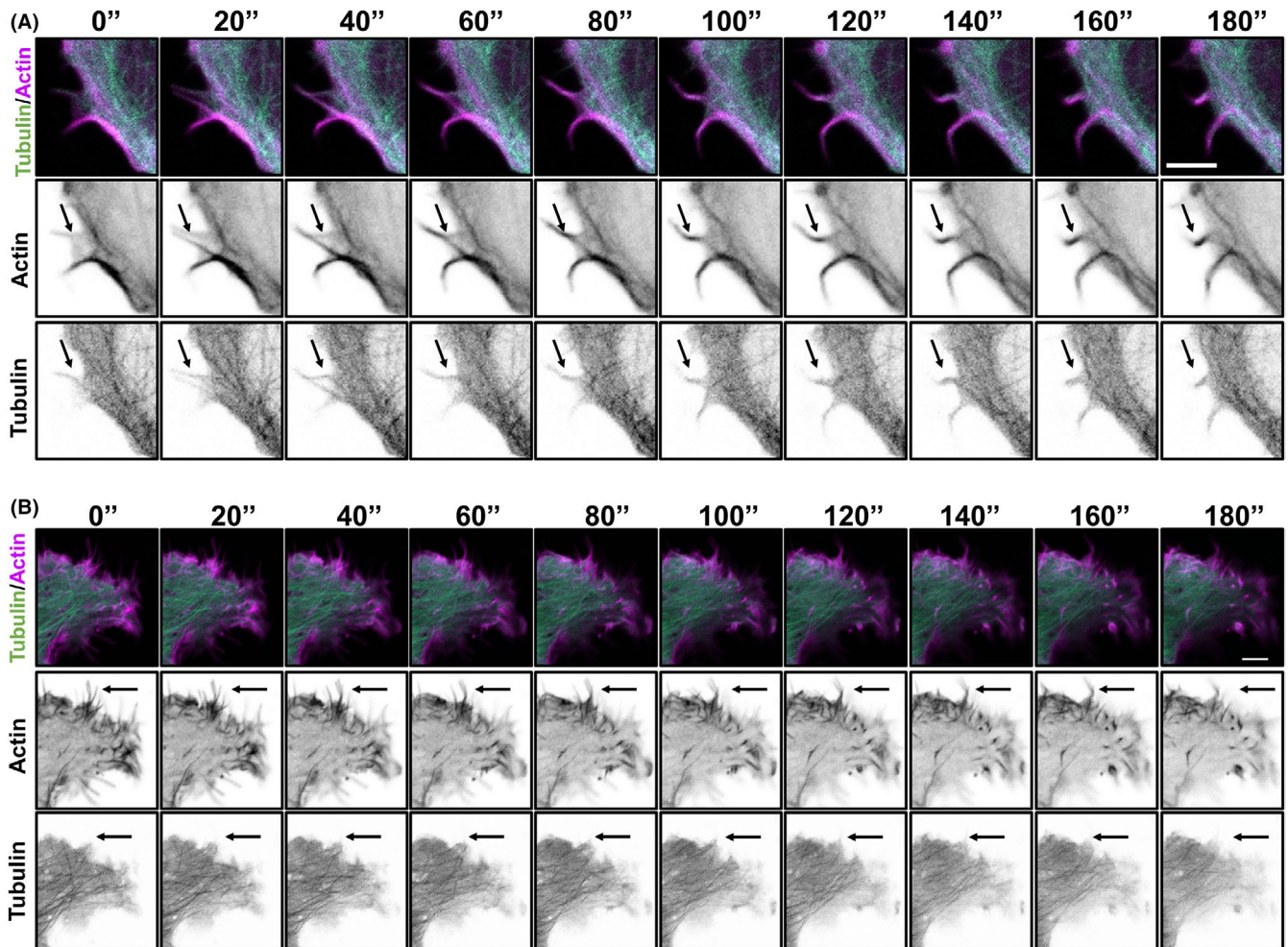


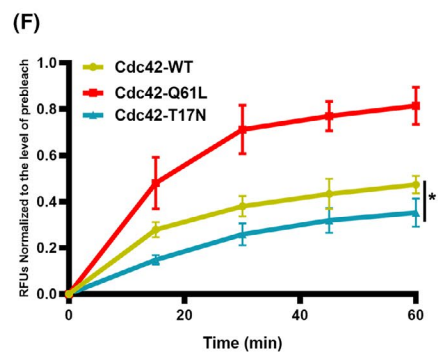
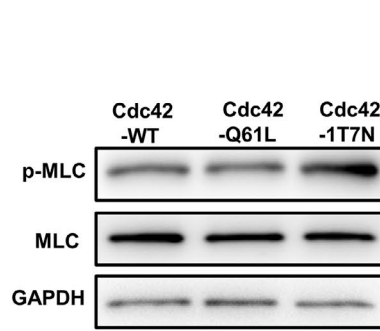
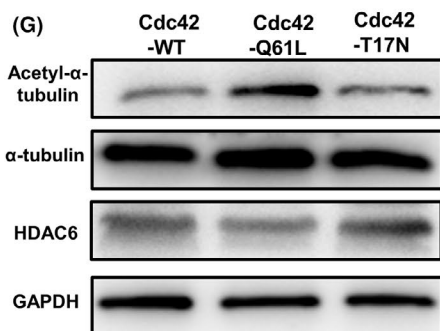
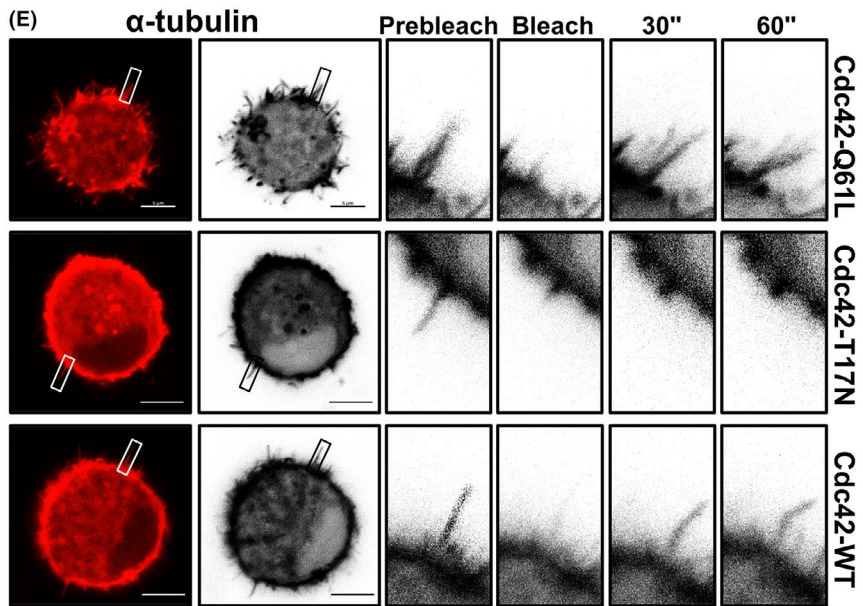
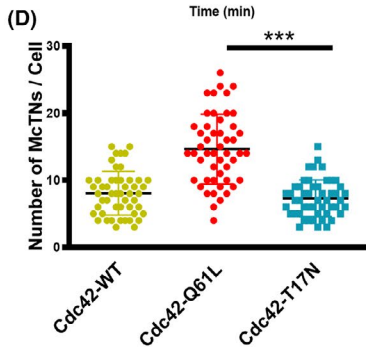
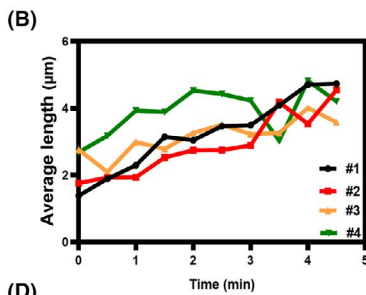
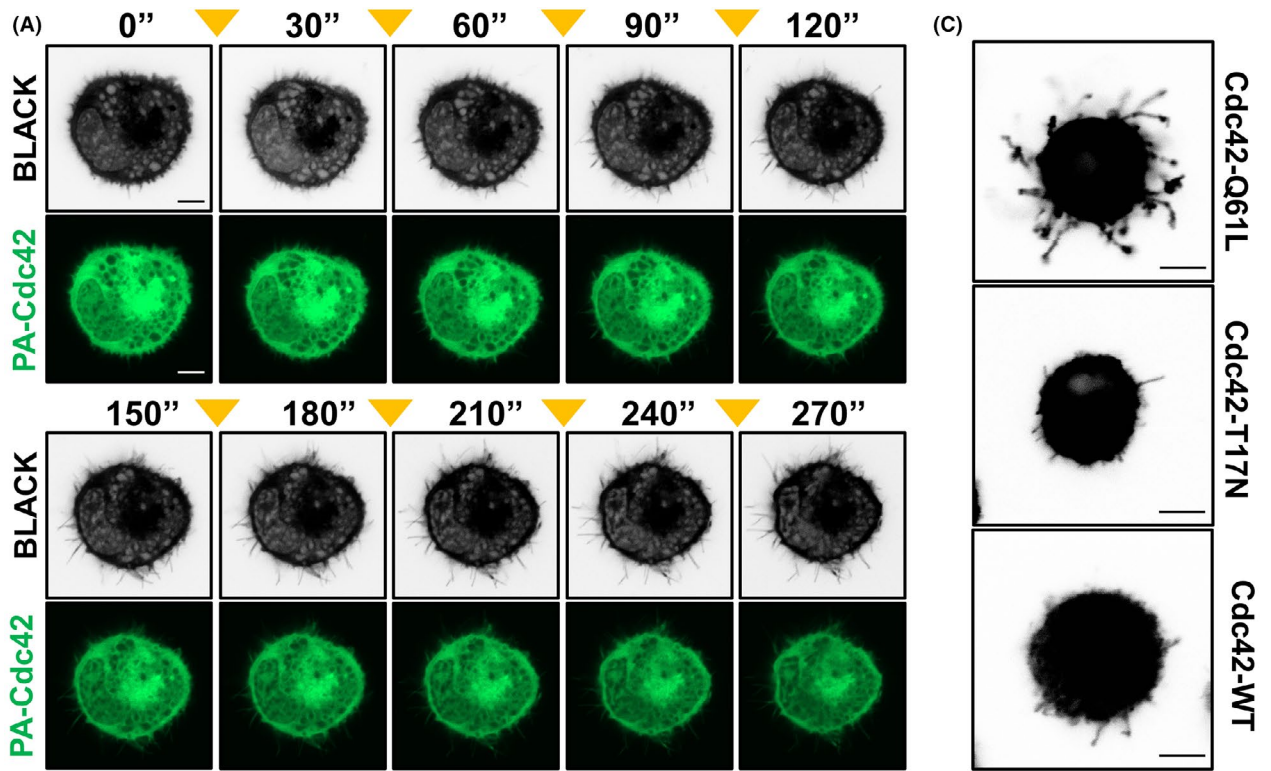
FIGURE 4 McTNs are dynamic and stabilized by a balance between microtubules and actin. **A**, Representative frames were cropped from time-lapse microscopy to show cytoskeleton dynamic. MDA-MB-231 cells were cultured for 24 h and then co-transfected with GFP-tubulin and mCherry-LifeAct. McTN dynamics was recorded using time-lapse microscopy for 3 min. Arrows show an example microtubule and actin microfilament in parallel; see also the Video S2. Scale bar = 5 μ m. **B**, Cells were co-transfected with GFP-tubulin and mCherry-LifeAct. Adherent cell morphology was observed using time-lapse microscopy. The arrow shows an example in which a microtubule is growing in parallel with actin. Scale bar = 5 μ m

4 | DISCUSSION

Complex signaling pathways involving the cancer cell and tumor microenvironment mediated cell invasion at the primary site, survival, and arrest in the bloodstream, and outgrowth at a new site.¹ Understanding these signaling pathways in malignant tumor

behavior will contribute to controlling this fatal disease. It was reported that Notch-1 signaling promoted cancer cell proliferation, phenotype transition, metastasis, and drug resistance, leading to a poor prognosis for patients.^{25,37,38} Among these processes, metastasis is the major cause of cancer-related death, and Notch-1 signaling is known to contribute to metastasis via multiple mechanisms.

FIGURE 5 Cdc42 activation-induced McTNs formation. **A**, Representative frames were cropped from time-lapse microscopy to observe the formation of McTNs. Confocal images of MDA-MB-231 cells expressing photoactivatable Cdc42 mutant (PA-Cdc42). See also the Video S3. The yellow triangles represent a photoactivation every 30 s for 5 min. Scale bars = 5 μ m. **B**, Cells were transfected with photoactivatable Cdc42 mutant (PA-Cdc42), then the variation trend of McTNs average length vs time was recorded by time-lapse microscopy for 5 min. **C**, Representative confocal images of McTNs protrusion. MDA-MB-231 cells were transfected with dominant active Cdc42 mutant (Cdc42-Q61L), dominant-negative Cdc42 mutant (Cdc42-T17N) or wild-type Cdc42 as a control, and the micrographs of suspended cells captured with confocal microscopy. Scale bars = 5 μ m. **D**, Some McTNs were carried out on suspended cells. $n = 50$ total cells. Values are means \pm SEM. **E**, Cells were subjected to FRAP analysis to examine the recovery of mCherry- α -tubulin fluorescence signaling after bleaching. **F**, The graph reports the average mobile fraction fluorescence intensity in the bleached zone during recovery. **G**, Expression of HDAC6, acetyl- α -tubulin, and p-MLC was examined by western blot assay



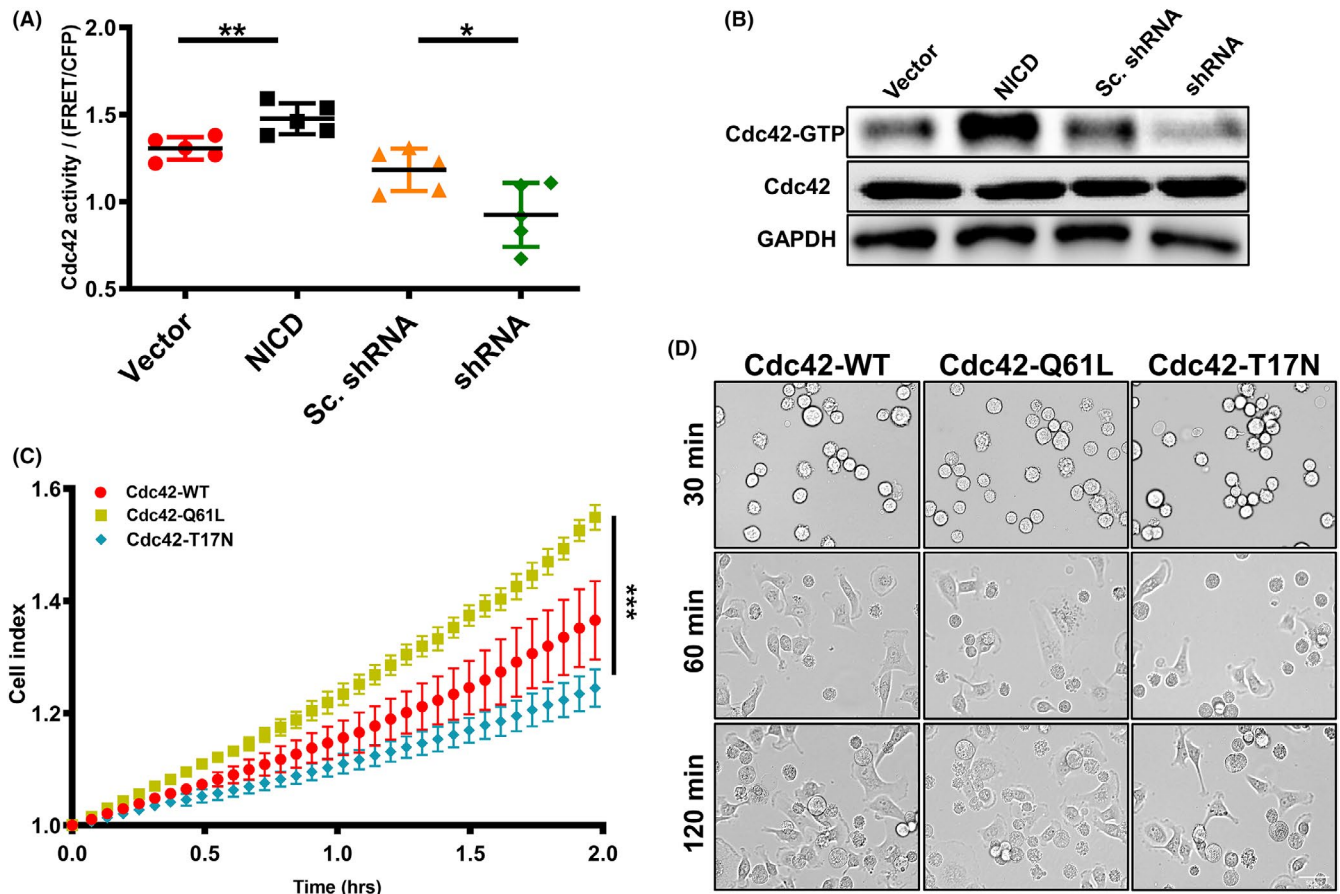


FIGURE 6 Notch-1 signaling activated Cdc42 to promote reattachment of suspended cancer cells. **A**, Cells were transiently transfected with YFP/CFP-tagged Cdc42 mutant. Basal FRET/CFP ratios of cells are plotted. Each dot corresponds to the value from a single cell, and at least 5 cells were analyzed. The horizontal bar indicates the mean. **B**, Western blot analysis was done to determine levels of Cdc42-GTP. **C**, Cells were transfected with Cdc42-WT, Cdc42-Q61L, and Cdc42-T17N, and then real-time cell reattachment was analyzed using the Electric Cell-Substrate Impedance Sensing (ECIS). Cell index represents the variation in electrical impedance over time. **D**, Representative phase contrast images of suspended cell reattachment. Cells were suspended in medium and incubated at 37°C. The images were acquired every 30 min for 2 h, Scale bar = 50 μ m

First, several findings suggested that Notch-1 signaling regulates aberrant expression of EMT markers such as Twist, Snail, and Slug to induce EMT.³⁹⁻⁴¹ Second, our previous studies have demonstrated that activation of Notch-1 signaling could promote cancer cell invasion and migration through NF- κ B activation via a PP2A-dependent AKT pathway.^{21,24} As is well known, the efficient arrest of CTCs is a key step for the establishment of metastatic foci.^{42,43} Cancer cells that exhibit mesenchymal and stem cell phenotypes promote McTN formation and reattachment, and recent evidence also suggested that invasive suspended tumor cells formed more McTNs to promote cell reattachment compared with non-invasive cell line cancer cells.⁶ In this context, it is informative to investigate whether Notch-1 signaling contributes to CTC reattachment and elucidate the underlying mechanisms. Here, our data showed that Notch-1 signaling activation triggered McTN formation to promote reattachment of suspended human breast cancer cells.

The perturbation of cytoskeletal filaments will affect McTN formation and dynamics. Therefore, we further explored the effect of rearrangement of microtubules and actin filaments on McTN

formation, respectively. We found that suspended cancer cells exhibited a significant increase in McTN formation after destabilizing actin filaments to destroy the inward contractility driven by the actin cortex. It may be due to the loss of a barrier to counterbalance the outward extension of microtubules. As actomyosin-driven contractility also plays an important role in McTN formation, we asked how Notch-1 signaling functions in actin filaments remodeling. Cofilin has been recognized as an actin-severing protein that is activated by dephosphorylation to induce F-actin depolymerization and is essential for cell migration.^{36,44} Unexpectedly, it was found that cofilin activity is not required for Notch-1-induced McTN formation. As cofilin is not the sole determinant of cell contractility, we wondered whether myosin II would affect McTNs formation. A previous study has revealed that inhibition of actomyosin contractility promoted the survival of suspended cells in fluid shear flow and induced a stem-like phenotype in primary cancer cells, and that obscurin downregulation could also decrease the phosphorylation of MLC to promote McTN formation.^{45,46} In this study, we demonstrated that Notch-1 signaling activation suppressed MLC activity to reduce cell contractility, and

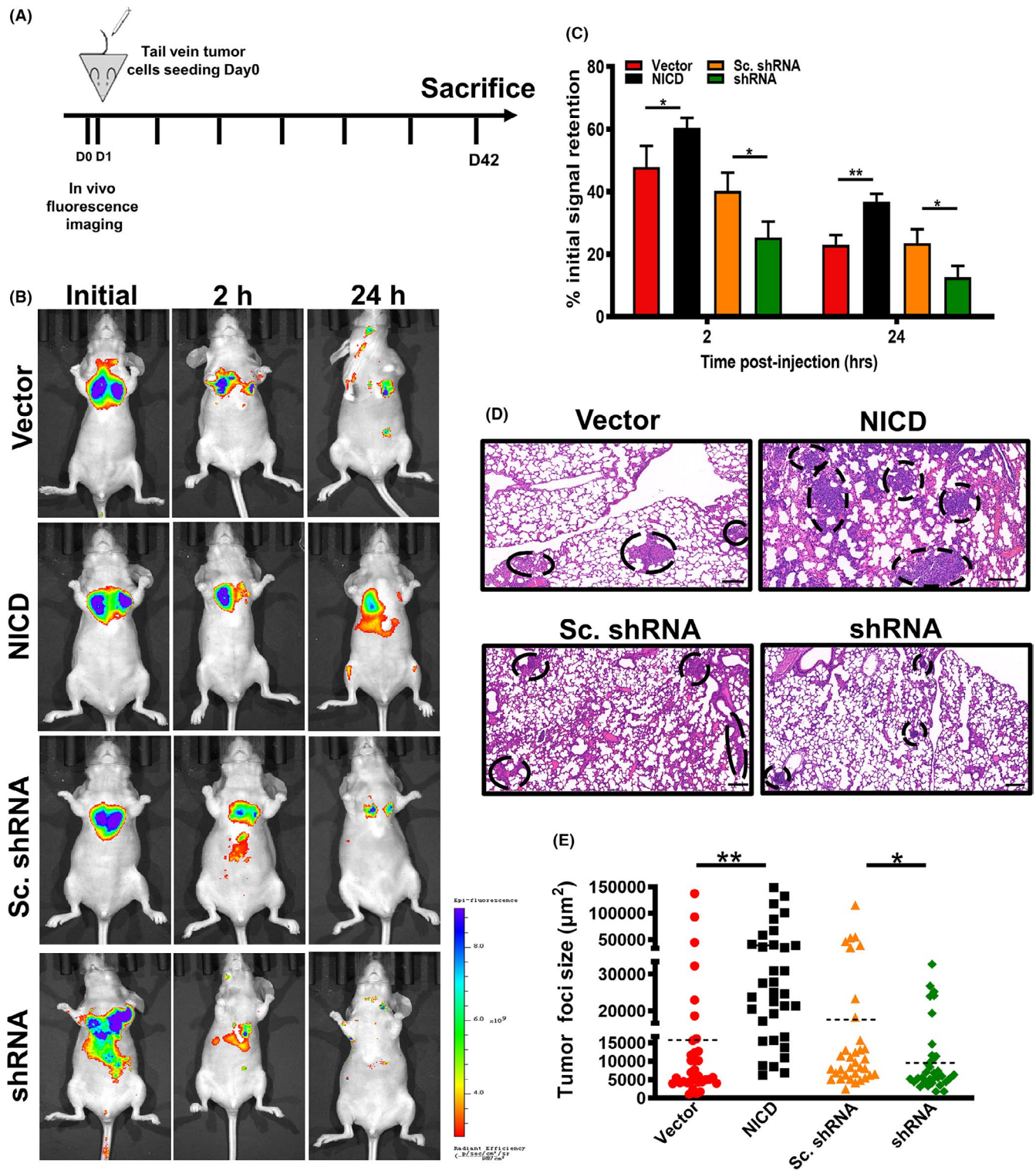


FIGURE 7 Notch-1 activation (NICD overexpression) promotes in vivo trapping and retention of circulating tumor cells in the microvasculatures. **A**, Experimental schedule for the retention assay of circulating tumor cells in lungs and tumor metastasis in the lungs. Cells that transfected with empty vector, NICD, Sc. shRNA, or shRNA were injected at day 0. In vivo fluorescence imaging was performed at 2 h and 24 h post-injection, and the mice were sacrificed at day 42. **B**, Fluorescence images of the arrested circulating tumor cells in the lung after intravenous injection at 2 h and 24 h. **C**, Retention of fluorescence signals was measured and represented as the percentage of initial peak signal intensities. Data represented as the mean \pm SD of 3 independent experiments. **D**, Representative images showing the lung nodules of sections. Lung sections were fixed and then stained with hematoxylin and eosin, and the tumor-covered areas were quantified. Scale bar = 200 μ m. **E**, Quantification of tumor sizes of lung areas positive for foci, $n = 34$. Values are means \pm SEM. * $P < .05$

that reducing cytoskeletal stiffness by inhibiting myosin II increased McTN formation. This finding also supported the fact that cytoskeleton softening was observed during cancer malignant progression, and assists cancer cells to squeeze through the extracellular matrix and extravasate through the vasculature to achieve distal metastasis.⁴⁷ In addition to modulating the actin cytoskeleton, Notch-1 signaling could also regulate microtubule organization through post-translational modifications such as acetylation. Our results verified that Notch-1 activation increased the expression of acetylated α -tubulin to stabilize microtubules, and led to extend long McTN formation. Microtubule stabilization will facilitate the EMT, cell migration, and is associated with poor prognosis in breast cancer.^{35,48}

A further study showed that suspended human breast cancer cells exhibited a high co-localization of microtubules and actin filaments in McTNs. We next questioned whether there was an interplay of microtubules and microfilaments in McTNs. Actin-microtubule crosstalk is important for the regulation of cell shape,

cell polarity, and migration. It has been reported that Cdc42 could coordinate microtubule and actin interaction through its target effectors and reinforce cell polarization and motility. In our study, we found that Notch-1 signaling upregulated Cdc42 activity to induce McTNs formation. Whether cdc42 regulated the interplay of microtubules and microfilaments in McTNs needs further investigation. In migrating cells, Cdc42 activity plays a definitive role in regulating filopodia formation and actin cytoskeletal dynamics. LIMK2 is a downstream effector of Cdc42 for actin cytoskeletal regulation and could phosphorylate cofilin under the control of Cdc42.⁴⁹ However, stated earlier, Notch-1 activation did not affect cofilin activity. We speculated that cofilin activity is a coordinated regulation by other molecules. Especially, phosphorylation of cofilin is also regulated by the RhoA pathway.⁵⁰ Furthermore, recent biosensor studies have shown that all 3 GTPases may regulate one another: Cdc42 can activate Rac1,^{51,52} and Rac1 and RhoA are mutually inhibitory.^{53,54} This indicates that Cdc42 and RhoA may operate antagonistically

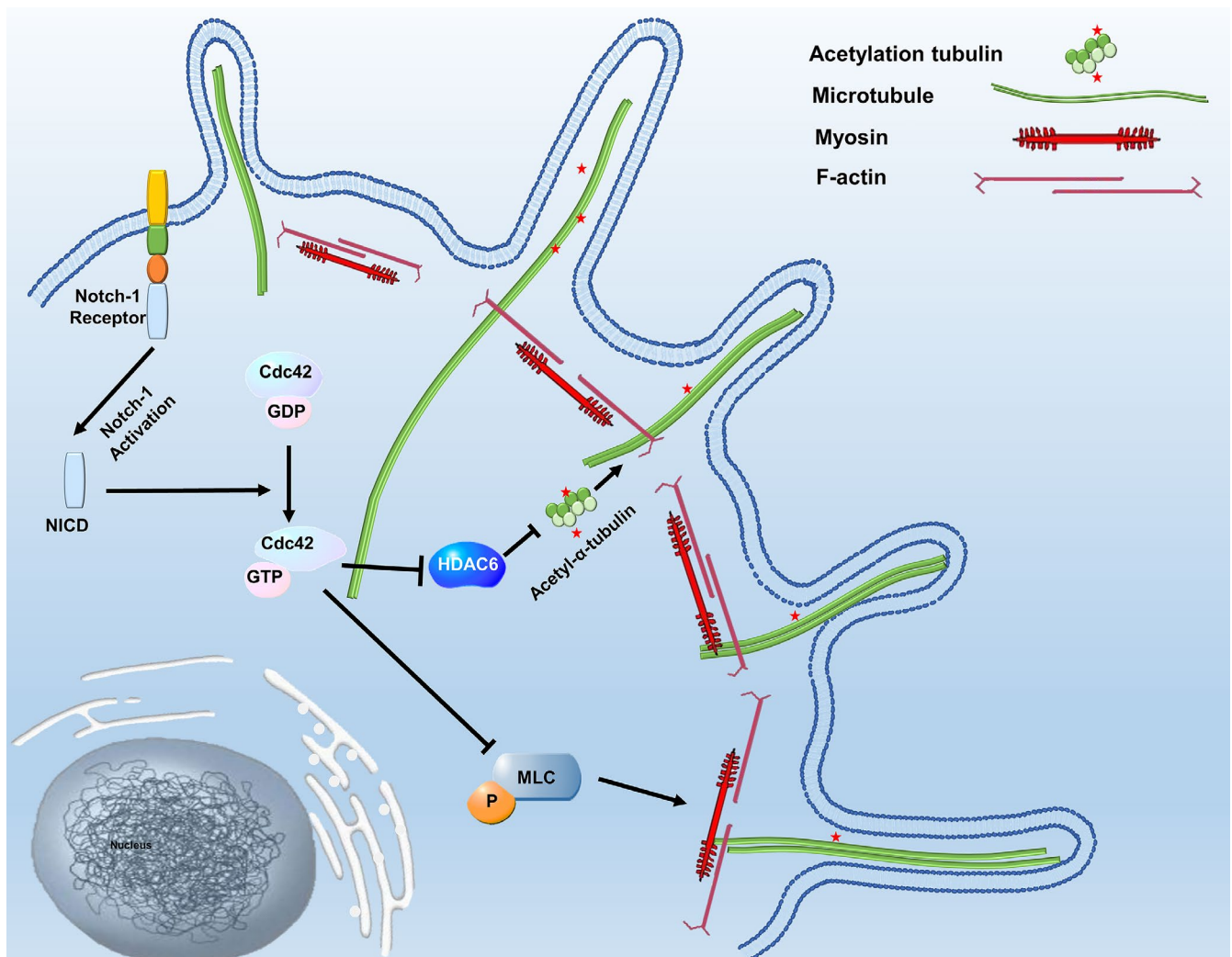


FIGURE 8 Schematic illustration of Notch-1 activation and the downstream signaling pathway for McTNs formation of suspended cancer cells. Notch-1 signaling triggers Cdc42-mediated McTNs formation to facilitate suspended cancer cells reattachment. The activation of Notch-1 signaling increased the polymerization of microtubule by the Cdc42/HDAC6 pathway as well as suppressed the actomyosin by Cdc42/MLC pathway, thereby promote the McTNs formation and cell reattachment. By this co ordination regulation, Notch-1 signaling plays a critical role in the balance between the outward extension of microtubules and inward contractility of actin in McTNs formation

in actin cytoskeleton dynamics. Cdc42 is a member of the small GTPases family that would be hyperactivated by signaling through oncogenic cell surface receptors, which converge on guanine nucleotide exchange factors to regulate their GDP/GTP switch.¹⁵ In this study, we found that Cdc42 acted as a signaling transduction molecule to trigger McTN formation and cell reattachment via the Notch-1 signaling pathway. The downstream molecules involved in the Cdc42-induced McTN formation included HDAC6 and p-MLC. Although our data indicated that the Notch-1/Cdc42 signaling axis mediated McTN formation, how Notch-1 regulates Cdc42 and its downstream signaling still need to be explored further. It has been reported that the PI3K-Cdc42-Pak-Mek-Erk signaling pathway regulates immune-complex-induced apoptosis in human neutrophils, and that Cdc42 is activated in a PI3K-dependent manner in this apoptosis process.⁵⁵ Furthermore, the PI3K catalytic subunit mutations result in hyperactivation of Cdc42 guanine nucleotide exchange factors and therefore elevate Cdc42 signaling.¹⁵ In addition, another report demonstrated that Notch-1 signaling would trigger the PI3K-AKT-mTOR pathway to regulate immature T-cell growth.⁵⁶ Our previous study has also confirmed that Notch-1 activation facilitated cell invasion and induced the crosstalk between PI3K/AKT and NF- κ B pathways.²⁴ Collectively, all these studies implied that PI3K might be involved in the cascades between Notch-1 and Cdc42 pathways.

In conclusion, our studies revealed the role of Notch-1 signaling in cell reattachment. Activation of Notch-1 signaling increased the polymerization of microtubules through the Cdc42/HDAC6 pathway, as well as suppressed actomyosin by the Cdc42/MLC pathway, thereby promoting McTN formation and cell reattachment. Through this coordination regulation, Notch-1 signaling plays a critical role in the balance between the outward extension of microtubules and inward contractility of actin in McTN formation (Figure 8).

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CONFLICT OF INTEREST

The authors claim no conflicts of interest.

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SUPPORTING INFORMATION

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