The Microtubule Plus End Tracking Protein TIP150 Interacts with Cortactin to Steer Directional Cell Migration*

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Cell migration is orchestrated by dynamic interactions of microtubules with the plasma membrane cortex. How these interactions facilitate these dynamic processes is still being actively investigated. TIP150 is a newly characterized microtubule plus end tracking protein essential for mitosis and entosis (Ward, T., Wang, M., Liu, X., Wang, Z., Xia, P., Chu, Y., Wang, X., Liu, L., Jiang, K., Yu, H., Yan, M., Wang, J., Hill, D. L., Huang, Y., Zhu, T., and Yao, X. (2013) Regulation of a dynamic interaction between two microtubule-binding proteins, EB1 and TIP150, by the mitotic p300/CBP-associated factor (PCAF) orchestrates kinetochore microtubule plasticity and chromosome stability during mitosis. J. Biol. Chem. 288, 15771–15785; Xia, P., Zhou, J., Song, X., Wu, B., Liu, X., Li, D., Zhang, S., Wang, Z., Yu, H., Ward, T., Zhang, J., Li, Y., Wang, X., Chen, Y., Guo, Z., and Yao, X. (2014) Aurora A orchestrates cell migration by regulating a dynamic MCAK-TIP150 interaction. J. Mol. Cell Biol. 6, 240–254). Here we show that TIP150 links dynamic microtubules to steer cell migration by interacting with cortactin. Mechanistically, TIP150 binds to cortactin via its C-terminal tail. Interestingly, the C-terminal TIP150 proline-rich region (CT150) binds to the Src homology 3 domain of cortactin specifically, and such an interaction is negatively regulated by EGFR-elicited tyrosine phosphorylation of cortactin. Importantly, suppression of TIP150 or overexpression of phospho-mimicking cortactin inhibits polarized cell migration. In addition, CT150 disrupts the biochemical interaction between TIP150 and cortactin in vitro, and perturbation of the TIP150-cortactin interaction in vivo using a membrane-permeable TAT-CT150 peptide results in an inhibition of directional cell migration. We reason that a dynamic TIP150-cortactin interaction orchestrates directional cell migration via coupling dynamic microtubule plus ends to the cortical cytoskeleton.

Cell migration is an essential physiological function that is tightly regulated in numerous biological processes, including development, tissue remodeling, wound healing, and tumor metastasis (1, 2). These processes require coordination of dynamic reorganization between the actin filaments and microtubules.

Cortactin (CTN)6 is a multidomain-containing scaffold protein that consists of an N-terminal acidic domain, six and a half tandem repeats, an α helix, a proline-rich region, and an Src Homology (SH3) domain at its C terminus (3). CTN is an important scaffold for promoting the polymerization and rearrangement of the actin cytoskeleton (4). Functionally, CTN, distributed at protrusions and the leading edges, is necessary for cell migration by regulating intercellular adhesion and cell spreading (5). Importantly, CTN is phosphorylated at tyrosine residues in response to growth factor receptors, which regulates cell migration (6).

Microtubules, one of the three major components of the cytoskeleton, are required for maintaining the physical and plastic properties of migrating cells (7). The plus ends of microtubules extend into the cell peripheral regions, dynamically switching between growing and shortening phases (8). The cytoskeletal interactions controlling cell migration are mediated by mechanisms that promote dynamic regulation of microtubule-associated proteins (MAPs) (9). Previous studies suggest that MAP-dependent regulatory pathways control cell migration by stimulating actin polymerization at the leading edge of migrating cells and, through the modulation of dynamic cell adhesion properties, regulate both the stabilization and disassembly of focal adhesion complexes (10). Focal adhesion

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6 The abbreviations used are: CTN, cortactin; SH3, Src homology 3; MAP, microtubule-associated protein; +TP, plus end tracking protein; EB, end-binding; PACF, photoactivatable complementary fluorescent; N-WASP, neuronal Wiskott-Aldrich syndrome protein.
complexes are initiated outside of the cell by binding to the extracellular matrix, where they mechanically connect the extracellular matrix to the actin cytoskeleton and provide the anchor points required for cell migration (1, 10, 11). Migrating cells organize a dynamic extracellular matrix, activate proto-oncogene tyrosine-protein kinase Src based on extracellular matrix stiffness, and synergize various organelle dynamics (12). One group of MAPs regulates microtubule dynamics and interactions within cells via their localization at the plus ends of microtubules, which are called microtubule plus end tracking proteins (+TIPs) (13). +TIPs have been identified based on their actual localization at the growing ends of microtubules and their regulation by virtual interactions with the end-binding (EB) family of +TIP proteins.

Our early studies revealed that TIP150 is an EB1-based microtubule plus end tracking protein necessary for accurate microtubule-kinetochore attachment during cell division (14, 15). Our recent work showed that TIP150 is required for dynamic microtubule-plasma membrane interaction during entosis (16). Linkage of microtubule plus ends with the cell cortex is required for directional cell migration. However, the functionality and mechanistic role of TIP150 in cell migration remain unclear.

In this study, we identify that TIP150 directly associates with the actin-binding protein, CTN, via its C-terminal proline-rich region (CT150). Interestingly, the CT150 associates with the SH3 domain of CTN, and the interaction is negatively regulated by EGF-elicited tyrosine phosphorylation of CTN. Moreover, suppression of either TIP150 or CTN inhibited directional cell migration. Perturbation of the TIP150-CTN interaction using a microtubule end-binding proteins, we carried out an affinity isolation of a TIP150-containing protein complex followed by mass spectrometric identification of tryptic peptides derived from the TIP150-Cortactin Interaction Orchestrates Cell Migration

**Results**

**Identification of a Novel TIP150-CTN Complex in Interphase Cells**—Our previous studies have revealed the functional importance of TIP150 in kinetochore and microtubule plus end dynamics during mitosis and entosis (14, 16). Because TIP150 protein levels remain unchanged during the cell cycle, we sought to examine the role of TIP150 in interphase cells. To study the molecular association of TIP150 with other microtubule end-binding proteins, we carried out an affinity isolation of a TIP150-containing protein complex followed by mass spectrometric identification of tryptic peptides derived from the individually sliced bands as described previously (17, 18). As shown in Fig. 1A, anti-TIP150 affinity beads covalently coupled to an anti-TIP150 antibody isolated a major polypeptide of 150 kDa in addition to three visible bands of polypeptides with approximate masses of 73, 55, and 25 kDa (lane 2). The 150-kDa polypeptide was absent from control IgG affinity matrix isolation (Fig. 1A, lane 1) and was confirmed as 150 kDa based on mass spectrometry and Western blotting analyses. Our mass spectrometric analyses identified the 73-kDa polypeptide as CTN, a characterized cortical actin-binding protein interacting with Grb2 in cell migration and cellular polarity establishment (19, 20). Western blotting analyses validated that the microtubule plus end protein EB1 also existed in the immunoprecipitates of TIP150, whereas the actin-binding protein ezrin was absent (Fig. 1B). Reciprocal immunoprecipitation using an anti-CTN antibody confirmed that TIP150 forms a cognate complex with CTN (Fig. 1C, lane 3, second panel). Ezrin was absent in the CTN immunoprecipitates (Fig. 1C, lane 3, third panel), suggesting that the CTN-TIP150 interaction is selective. As a positive control, Grb2 was retained by CTN immunoprecipitation (Fig. 1C, lane 3, fourth panel). Consistently, the TIP150-CTN interaction also occurs in MCF7 and HeLa cells (Fig. 1, D and E). Thus, we conclude that TIP150 forms a cognate complex with CTN in interphase cells.

The cortical cytoskeleton constitutes an important subcellular structure that determines cell shape and orchestrates cellular dynamics. CTN is an actin-binding protein located at the cortical cytoskeleton for integration of diverse signaling pathways. The identification of CTN in the TIP150 protein complex prompted us to examine the distribution profiles of TIP150 and CTN in interphase cells. To validate the hypothesis, we carried out immunofluorescence microscopic analyses of MDA-MB-231 cells triple-stained for CTN (red), TIP150 (green), and microtubules (blue). As shown in Fig. 1F, b’, green, TIP150 distributes in the cytoplasm of interphase MDA-MB-231 cells and appears as comet-like structures, reminiscent of microtubule plus ends. Careful examination revealed a brief localization of TIP150 around plasma membrane regions reminiscent of membrane ruffle structures (Fig. 1F, b’, arrowheads). Surprisingly, this membrane ruffle-like localization of TIP150 is partially superimposed onto that of CTN signal (Fig. 1F, red), as shown in the magnified montage of triple color merged images (Fig. 1F, arrowheads). Thus, we conclude that TIP150 interacts and co-distributes with CTN near the plasma membrane in interphase cells.

**Characterization of the Physical Interaction between TIP150 and CTN**—CTN is a signaling scaffold molecule containing several structural modules that facilitate the orchestration of protein-protein interactions underlying the signaling cascade during cell polarity establishment and cell migration. On the other hand, TIP150 also contains structural determinants essential for interacting with EB1 (14). We hypothesized that TIP150 physically interacts with CTN and that the binding interface and perhaps structural determinants could be uncovered by biochemical characterization. To test this hypothesis, we designed a series of deletion mutants of TIP150 and CTN according to their structural features (Fig. 2, A and Fig. B) and expressed those proteins tagged with GFP in HEK293T cells.

To first determine the specificity of TIP150-CTN interaction, aliquots of HEK293T cells were transiently transfected to express FLAG-TIP150 with GFP-tagged IQGAP1 (GFP-IQ1), N-WASP (GFP-WASP), CTN (GFP-CTN), and GFP alone. Anti-FLAG immunoprecipitation using M2 affinity matrix followed by anti-GFP blotting analyses confirmed that FLAG-TIP150 forms a cognate complex with GFP-CTN (Fig. 2C, lane 7, top panel). Neither IQGAP1 nor N-WASP was present in the FLAG-TIP150 immunoprecipitates (Fig. 2C, lanes 5 and 6, top panel), confirming that the CTN-TIP150 interaction is selective.
To further evaluate the physical interaction and pinpoint the regions of TIP150 involved in the interaction, we carried out a pulldown assay using GST-CTN as an affinity matrix to absorb the GFP-TIP150 and its deletion mutants from HEK293T cell lysates. As shown in Fig. 2D, right panel, lanes 15–18, Western blotting analyses with a monoclonal antibody of GFP demonstrated that the GFP fusion proteins containing the C-terminal TIP150, in addition to full-length GFP-TIP150, were readily retained on GST-CTN matrix. The N-terminal domain also exhibited weak binding activity toward CTN (Fig. 2D, right panel, lane 12). To pinpoint the precise region of CTN involved in TIP150 binding, we expressed recombinant GST-CTN and its deletion mutants in bacteria. Purified GST-CTN proteins (full-length, N terminus, and C terminus) were used as affinity matrices to absorb the GFP-TIP150 from HEK293T cell lysates. As shown in Fig. 2E, top panel, lanes 6–7, Western blotting analysis with an anti-GFP antibody indicated that the TIP150 binds to the C-terminal domain of CTN. The Coomassie Brilliant Blue-stained gel showed the quality of purified GST-CTN proteins (Fig. 2E, bottom panel).

To define the exact domain(s) essential for their physical interaction, we carried out an additional GST pulldown assay using fine deletion mutants of CTN as affinity matrices to absorb GFP-TIP150 from HEK293T cell lysates. Immunoblotting with an anti-GFP antibody confirmed that GFP-TIP150 was retained on GST-CTN, indicating a direct interaction between the CTN SH3 domain and the TIP150 C-terminal (amino acids 898–1110) in vitro (Fig. 3D, lane 21). The EB1-binding domain alone exhibited no detectable binding activity toward CTN protein (Fig. 3D, lane 17). Thus, we conclude that TIP150 directly interacts with the CTN SH3 domain through its C-terminal coiled-coil domain.
Phosphorylation of CTN at Its SH3 Domain Regulates CTN-TIP150 Interaction—Phosphorylation of CTN regulates its activity, including its ability to promote actin polymerization and cell migration (21). In particular, CTN is phosphorylated on tyrosines 421, 466, and 482 in response to EGF stimulation (22). Cell motility could be inhibited by truncating the N-terminal F-actin binding domains of CTN or by blocking tyrosine phosphorylation mutation sites (Y421F/Y466F/Y482F, non-phosphorylatable) (23). Next we tested whether EGF stimulation modulates TIP150-CTN interaction in cells. MDA-MB-231 cells were stimulated with EGF (100 ng/ml) for 5 min after serum starvation of 8 h. The cell lysates were then incubated with TIP150 affinity matrix beads. The results showed that the TIP150-CTN association in MDA-MB-231 cells was reduced by EGF stimulation (Fig. 3E). Interestingly, mutation of CTN phosphorylation sites (Y421E/Y466E/Y482E, phosphomimetic mutants) have been shown to reduce the ability to interact with focal adhesion kinase and promote cell migration (23). We sought to investigate whether mutations at these phosphorylation sites had any effect on TIP150 interaction. To determine whether the phospho-mimicking mutants modulate the TIP150 interaction with CTN, we used anti-GFP antibody to
probe GFP-TIP150 bound to the affinity beads preloaded with GST-CTN and a triple site mutation: GST-CTN<sup>Y421F/Y466D/Y482F</sup> (Fig. 3F, 3Y/F) and GST-CTN<sup>V421D/Y466D/Y482D</sup> (Fig. 3F, 3Y/D). Quantitatively, the relative binding efficiency of TIP150 was significantly less, <i>p</i> < 0.001, when tyrosine was changed to aspartic acid, mimicking the phosphorylated form of CTN (Fig. 3G), suggesting that reversible phosphorylation may regulate the dynamic interaction between TIP150 and CTN.

The C-terminal region of CTN contains a series of tyrosine residues: 421, 466, 482, 497, 499, and 541, which are conserved between mouse and human (24). To further evaluate the effect of tyrosine phosphorylation of CTN on the TIP150-CTN interaction, we carried out another pull-down assay using GST-CTN phosphorylation mutants as affinity matrices. As shown in Fig. 3H, FLAG-TIP150 from HEK293T cell extracts directly bound to GST-CTN<sup>Y497F</sup> but not to GST-CTN<sup>Y497D</sup> or GST-CTN<sup>Y541D</sup>. The binding of TIP150 was reduced by half. GST-CTN<sup>Y497D</sup> and GST-CTN<sup>Y541D</sup> virtually lost their binding to TIP150 (Fig. 3I), suggesting that these sites are major residues essential for TIP150-CTN interactions.

**TIP150 Participates in Directional Cell Migration**—Several microtubule plus end tracking proteins have been reported to modulate cell migration by different mechanisms (7, 13). Our immunofluorescence microscopic analyses show that TIP150 co-distributes with CTN near the plasma membrane in interphase cells (Fig. 1F). To examine the possible function of endogenous TIP150 and TIP150-CTN interactions in cell migration, we sought to suppress TIP150 and CTN in MDA-MB-231 cells using the indicated shRNAs. Because EB1 is involved in cell migration (25), we included EB1 as a positive control. Western blotting analysis revealed that TIP150 was efficiently depleted by specific shRNAs but not by scrambled sequences, whereas the protein levels of tubulin and EB1 were unaffected (Fig. 4, lanes 6–8). Quantitative analyses showed that GST-CTN<sup>Y497D</sup> binding of TIP150 was reduced by half. GST-CTN<sup>Y497D</sup> and GST-CTN<sup>Y541D</sup> virtually lost their binding to TIP150 (Fig. 3I), suggesting that endogenous CTN and TIP150 are regulators responsible for the EGF-stimulated cell migration.

The TIP150-CTN Interaction Is Essential for Cell Migration—To further validate our results at the single-cell level, MDA-MB-231 cells transfected with mCherry-tagged control shRNA or TIP150 shRNA were starved and then stimulated by serum to migrate. Suppression of TIP150 induced a measurable defect in cell migration (Fig. 5A, bottom panels). To quantify these results, the migration speeds of cells transfected with control shRNA or TIP150 shRNA were calculated. The quantitative analysis showed that TIP150 knockdown reduced the velocity of MDA-MB-231 cells by roughly 55% of that observed in control cells (Fig. 5B). We next examined the migration distance in the absence of TIP150. As shown in Fig. 5C, the total distance of travel from initial to final point upon stimulation with EGF was statistically different, whereas shRNA-TIP150-transfected cells migrated a shorter distance compared with the non-transfected group. Quantitative analyses showed that the distance of cell migration in the TIP150-depleted cells was half of that in control cells (Fig. 5C, <i>p</i> < 0.0001). Our quantitative analyses also show that shRNA-TIP150 significantly reduced the directional velocity of cell migration (Fig. 5, D–F). Thus, we conclude that TIP150 is required for directionally persistent cell migration in MDA-MB-231 cells.
As microtubule plus end dynamics in the cell cortex region are involved in cell morphogenesis, polarity, and migration, we focused on the role of TIP150 on microtubule plus end dynamics in this region. As shown Fig. 5F, TIP150 knockdown increased the unstable microtubule ends in the cell cortex region. In TIP150-depleted cells, the microtubule plus end growth rate and shrinking rate increased by 42.6% and 67.6%, respectively, relative to that in control cells (Fig. 5, H and I).

At the cortex of TIP150 knockdown cells, microtubule plus ends displayed a shorter time in pausing but a longer time in growing and shortening (Fig. 5J). The data suggest that TIP150 is likely responsible for microtubule stabilization in the cortex region.

The biochemical characterization of the TIP150-CTN physical interaction prompted us to investigate whether TIP150 cooperates with CTN in linking microtubule plus ends to the
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A 0 min 30 min 60 min 100 min 160 min 220 min 240 min
Ctrl shRNA
TIP150 shRNA

B

C

D

E

F

T: Total Distance
D: Directional Distance
Vf: Ti/Δt
Vg: Ti/Δt

G

H

I

J

K

L

M

N

O

P

Q

Vf (μm/min)

Total Velocity

0.25

0.20

0.15

0.10

0.05

0.00

Scramble
shTIP150
shCTN

Total Distance (μm)

Scramble
shTIP150
shCTN

Directional Distance (μm)

Scramble
shTIP150
shCTN

GFP-tubulin

shortening
pausing
growing
plasma membrane cortex. To this end, we first examined whether CTN alters TIP150-dependent microtubule plus end tracking in vitro using a total internal reflection fluorescence microscopy (TIRFM)-based assay reported previously (15, 20). Analyses of kymographs showed that wild-type TIP150 tracked the growing ends of microtubules in the presence of EB1. Addition of CTN did not alter the TIP150-dependent microtubule plus end tracking.7 Statistical analyses indicated that CTN did not modulate the TIP150-dependent microtubule dynamics. We next sought to examine whether TIP150 alters CTN function to modulate actin dynamics using the pyrene-actin assay reported previously (23). CTN promotes actin filament elongation. However, addition of TIP150 did not alter CTN-dependent actin filament elongation.⁹ Thus, these experiments show that TIP150 does not alter CTN function to modulate actin dynamics and that CTN does not modulate TIP150-dependent microtubule dynamics.

Next we examined the respective roles of TIP150 and CTN in cell migration. As shown in Fig. 5, K–M, suppression of CTN perturbed the trajectory in a similar pattern as seen in TIP150-depleted cells (Fig. 5L). Quantitative analysis showed that TIP150 knockdown reduced the velocity of MDA-MB-231 cells to roughly 60.9% and that CTN suppression reduced the velocity of MDA-MB-231 cells to roughly 39.6% compared with that of cells transfected with scramble shRNA (Fig. 5N). Further analyses of cell migration distances demonstrated that suppression of CTN resulted in a dramatic reduction of persistent migration (Fig. 5O). In addition, our quantitative analyses also showed that suppression of TIP150 or CTN significantly impaired the directional velocity of cell migration (Fig. 5, P and Q, p < 0.0001). Thus, both CTN and TIP150 are essential for EGF-elicted cell migration in MDA-MB-231 cells.

To assess the precise function of TIP150-CTN interaction in EGF-elicted cell migration, we designed a competitive peptide that would specifically perturb TIP150 binding to CTN. Consequently, we engineered a membrane-permeable peptide containing the PXXP motif of TIP150, which is involved in binding to CTN. This was achieved by introducing an 11-amino acid peptide derived from the TAT protein transduction motif into a fusion protein containing amino acids 898–1110 of TIP150, named TAT-GFP-CT150, that was involved in the binding interface between TIP150 and CTN (Fig. 3D). The recombinant protein was histidine-tagged and purified to homogeneity using nickel affinity beads (Fig. 6A). As predicted, the recombinant TAT-GFP-CT150 peptide disrupted the TIP150-CTN association in vivo, as judged by anti-TIP150 immunoprecipitation (Fig. 6B, lane 6). As a negative control, TAT-GFP did not interfere with the interaction of TIP150-CTN (Fig. 6B, lane 5).

MDA-MB-231 cells were treated with TAT-GFP or TAT-GFP-CT150 peptide for 30 min before imaging cell migration elicited by EGF; real-time imaging of the cells began 5 min after EGF addition. The migration tracks illustrated that treatment of cells with the TAT-GFP-CT150 peptide perturbed EGF-elicted cell migration relative to cells treated with the TAT-GFP control (Fig. 6, C and D). Our quantitative analyses show that the TAT-GFP-CT150 peptide significantly reduced the directional velocity of cell migration (Fig. 6, E–I). In addition, TAT-GFP-CT150-treated cells increased the unstable microtubule ends in the cell cortex region (Fig. 6J), suggesting that the TIP150-CTN interaction promotes microtubule dynamics. In TAT-GFP-CT150-treated cells, the microtubule growth rate and shortening rate increased by 37.8% and 60.2%, respectively, relative to that in control cells (Fig. 6, K and L). At the cortex of TAT-GFP-CT150-treated cells, microtubule plus ends displayed a shorter time in pausing but a longer time in growing and shortening (Fig. 6M). The data suggest that TIP150 may be responsible for microtubule stabilization in the cortex region.

To ensure that the observed cell crawling phenotype is not simply due to an imbalance in SH3-binding tyrosine kinase activity caused by an abundance of a PXXP-containing peptide, we mutated the PGYP-PPKP sequence of the TAT-GFP-CT150 peptide into PAVP-PARP. As shown Fig. 6N, PGYP-PPKP containing the TAT-GFP-CT150 peptide did not reduce the cell speed and directional velocity of cell migration. Thus, the TIP150-CTN interaction, mediated by the PGYP-PPKP motif, is essential for directional cell migration. We conclude that the TIP150-CTN interaction provides a regulatory linkage between the microtubule plus ends and cell cortex to steer cell migration.

**Discussion**

The highly regulated process of cell migration is critical for morphogenesis, tissue homeostasis, and tumor metastasis. Microtubule plus end tracking proteins constitute a complex structural platform that orchestrates the molecular events involved in regulating microtubule dynamics required for cell

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7 J. Zhou and X. Ding, unpublished observations.

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**FIGURE 5. TIP150 and CTN are essential for directional cell movement. A, MDA-MB-231 cells transfected with control or TIP150 shRNA (red) were treated as described under “Materials and Methods,” and images were collected at 10-min intervals. Ctrl, control. B, migration rates of TIP150-suppressed MDA-MB-231 cells were measured, and statistical significance was determined using Student’s t test. ***, p < 0.001. C, quantitative analysis measuring total migration distance of control or TIP150 shRNA-treated groups. Data represent mean ± S.D. and derived from 65 cells collected from three independent experiments. Statistical significance was determined by Student’s t test. ***, p < 0.001. D, quantitative analysis of relative cell speed. Data represent mean ± S.E. of 65 cells collected from three independent experiments. Statistical significance was determined by Student’s t test. ***, p < 0.001. E, quantitative analysis of directional distance. Data represent mean ± S.E. n = 65. ***, p < 0.001 by t test. F, visual demonstration of path length. The total distance between starting and ending points (I) and the actual trajectory (D) are indicated. G, TIP150 is required for microtubule plus end stabilization in the region of the cell cortex. Time-lapse images show the microtubule plus end dynamics in TIP150 knockdown cells. MDA-MD-231 cells were co-transfected with GFP-tubulin and control or TIP150 shRNAs for 72 h. Live-cell images were collected at 10-s intervals. Scale bar = 5 μm. H and I, statistical analysis of microtubule growth rate and shortening rate at the cell cortex in F. Data are presented as mean ± S.D. and derived from ~100 microtubules in 25 cells. ***, p < 0.001 by t test. J, the percentages of growing, pausing, and shortening times microtubules displayed at the cell cortex in F. Data are derived from ~100 microtubules in 25 cells. K–M, migration tracks of the indicated groups are presented from at least 20 cells. Data were collected from three independent experiments. N, quantitative analysis of total velocity in K–M. Data represent mean ± S.E. from three independent experiments. Statistical significance was determined by Student’s t test. ***, p < 0.001. O, quantitative analysis of measured total distance in K–M. ***, p < 0.001 by t test. P, quantitative analysis of directional distance in K–M. Data represent mean ± S.E. from three independent experiments. Statistical significance was determined by Student’s t test. ***, p < 0.001. Q, quantitative analysis of measured speed rates in K–M. ***, p < 0.001 by t test.
migration (13, 27). Directional cell movement is orchestrated by extracellular cue gradients; however, the molecular mechanism of microtubule dynamics regulated by unique +TIPs to mediate directional cell movement has remained elusive. TIP150 is a novel microtubule plus end tracking protein involved in various cellular processes (14–16). Previous studies have shown that many of the +TIPs accumulating at the cell cortex are involved in regulating cell migration through different pathways (13). In this study, we explored the physiological function of TIP150 and its association with cytoskeletal pro-

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FIGURE 6. Perturbation of the TIP150-CTN interaction inhibited cell migration. A, Coomassie Brilliant Blue (CBB) staining of SDS-PAGE gel showed the quality and quantities of the purified TAT-GFP-His6-tagged proteins. Bacteria expressing TAT-GFP-peptide (TAT-GFP-CT150) and TAT-GFP-His6 (TAT-GFP) were purified with nickel-nitrilotriacetic acid affinity chromatography and desalted into L-15. B, TIP150 immunoprecipitates from MDA-MB-231 cells incubated with TAT-GFP or TAT-GFP-CT150 peptide in interphase were immunoblotted with TIP150, CTN, EB1, and GFP antibodies. Nonspecific IgG-coupled beads were used as a control. Note that the EB1-TIP150 interaction was not altered by addition of the TAT-GFP-CT150 peptide but that the endogenous TIP150-CTN interaction was perturbed by addition of the TAT-GFP-CT150 peptide. IP, immunoprecipitation; HC, heavy chain. C and D, MDA-MB-231 cells were treated with the TAT-GFP or TAT-GFP-CT150 peptide for 30 min. The 5-h tracks of five randomly picked cells are presented for each group. E, visual demonstration of path length. The total distance between starting and ending points (T) and the actual trajectory (D) are indicated. F and G, quantitative analyses of total distance and directional distance. Data represent mean ± S.E. n = 35 and 29, respectively. **, p < 0.01; ***, p < 0.001. H and I, quantitative analyses of cell speed and velocity. Data represent mean ± S.E. n = 35 and 29, respectively. **, p < 0.01; ***, p < 0.001. J, the TIP150-CTN interaction is required for microtubule plus end stabilization in the region of the cell cortex. Time-lapse images show the microtubule plus end dynamics in mCherry-tubulin-expressing cells, which were treated with the TAT-GFP or TAT-GFP-CT150 for 30 min, and then live-cell images were collected at 10-s intervals. TAT-GFP-CT150-treated cells show microtubule retraction and shortening at the cell cortex in J. Data are presented as mean ± S.D. and derived from approximately 100 microtubule bundles from 25 cells. **, p < 0.01 by t test. M, the percentages of growing, pausing, and shortening times microtubules displayed at the cell cortex in F. Data are derived from ~100 microtubule bundles from 25 cells. N, MDA-MB-231 cells were treated with the TAT-GFP-CT150 (PGYP-PPKP) or TAT-GFP-CT150 mutant (PAPV-PARp) for 30 min. Presented are quantitative analyses of total distance and directional distance for 5 h. Data represent mean ± S.E. n = 36, ns, non-significant. Also presented are quantitative analyses of cell speed and velocity. Data represent mean ± S.E. n = 36.

FIGURE 7. Proposed working model accounting for the TIP150-CTN interaction in directional cell migration. TIP150 functions as a microtubule plus end stabilizer that interacts with CTN to promote microtubule-actin cortex interaction. EGF stimulation promotes phosphorylation of CTN, which accelerates a dynamic TIP150-CTN interaction at the leading edges of migrating cells for directional movement. Thus, the TIP150-CTN complex serves as a link to orchestrate directional cell migration via coupling dynamic microtubule plus ends with the cell cortex.

Polarized dynamics and organization of the cytoskeleton are required for directional cell migration. Microtubules are organized by the centrosome, where many are anchored by the minus end and extended into the periphery by the plus end. These pioneering microtubules fortify the interactions with actin. It has been shown that microtubules could be captured and guided by stiff actin bundles regulating microtubule-actin interactions along the cell cortex (31). The leading edge lamellipodium protrusion relies on continuous polymerization of actin into a dense, cross-linked meshwork of filaments that moves away from the cell edge when polymerized in a process called retrograde flow (32). CTN is an actin-binding protein and was initially identified as an Src substrate (33). CTN is phosphorylated by the tyrosine kinase Src in growth factor signaling, transformation, and pathogen invasion (22, 34–36). Several studies have suggested that CTN and phosphorylated CTN dynamically regulate lamellipodial protrusion, cell spread-

TIP150-Cortactin Interaction Orchestrates Cell Migration

FIGURE 7. Proposed working model accounting for the TIP150-CTN interaction in directional cell migration. TIP150 functions as a microtubule plus end stabilizer that interacts with CTN to promote microtubule-actin cortex interaction. EGF stimulation promotes phosphorylation of CTN, which accelerates a dynamic TIP150-CTN interaction at the leading edges of migrating cells for directional movement. Thus, the TIP150-CTN complex serves as a link to orchestrate directional cell migration via coupling dynamic microtubule plus ends with the cell cortex.
ing, intercellular adhesion, and cell motility. In addition, phosphorylation of CTN may enhance its ability to act as an adaptor or negatively regulate its function. Moreover, phosphorylation of CTN tyrosine is regulated by growing microtubules (28, 30, 34). This study suggests that EGF-elicited dissociation of the TIP150-CTN complex is needed to accelerate the recycling of TIP150 and CTN to promote leading edge membrane expansion and persistence microtubule growth, respectively. Thus, it would be of interest in the future to elucidate the structural basis of the TIP150 interaction with CTN. Elucidation of the structural basis of this interaction will enable us to consolidate the TIP150-CTN interaction and regulation into a mechanistic model by which the EGF-elicited tyrosine phosphorylation regulates the localization of microtubule plus ends to the cortical actin network.

Recently, we developed a new method for optically imaging intracellular protein interactions at a nanometer scale of spatial resolution in live cells using photoactivatable complementary fluorescent (PACF) proteins (37) based on photoactivation of complementary fluorescent proteins. Upon maturation, sparse subsets of PACF molecules were activated, localized, and then bleached (38). The aggregate position information from all PACF subsets was then assembled into a nanoscale map that allows imaging of a single-molecule copy of specific target protein-protein interactions in space and time in fixed and live cells. Using PACF, we obtained the precise localization of dynamic microtubule plus end hub protein EB1 dimers and their distinct distributions at the leading edges and cell bodies of migrating cells (37). Given the fact that TIP150 functions as dimers (15), in the future it would be of great interest to employ PACF-based analyses to probe how TIP150 dimers are precisely located to CTN in leading and trailing edges of migrating cells and whether alteration of the PXXP motif in a single TIP150 molecule of a dimer is sufficient to alter the TIP150-CTN linkage in cell migration at the single-molecule level. In addition, we can employ PACF to study the assembly and disassembly of the TIP150-CTN complex on a single microtubule and quantify how EGF-stimulated cell migration dynamics are correlated to spatial and temporal turnover of the TIP150-CTN complex.

In summary, we present evidence that TIP150 directly interacts with CTN. Our results also demonstrate that the TIP150-CTN interaction promotes microtubule dynamics and orchestrates EGF-elicited directional cell migration. However, more sophisticated mechanisms need to be delineated regarding how the reversible phosphorylation/dephosphorylation cycle regulates the aforementioned interaction dynamics during cell migration. Furthermore, it would be exciting and challenging to visualize and compare the single microtubule dynamics and corresponding cortex during cell migration.

Materials and Methods

Cell Culture—HEK293T cells obtained from the ATCC (Manassas, VA) were maintained as subconfluent monolayers in advanced DMEM (Gibco) with 10% FBS (HyClone), 100 units/ml penicillin, and 100 µg/ml streptomycin (Gibco) at 37 °C with 8% CO₂. MDA-MB-231 cells (ATCC) were maintained in L-15 medium (Gibco) containing 10% FBS at 37 °C. MDA-MB-231 cells were transfected with Lipofectamine 2000 (Invitrogen) according to the protocol of the manufacturer.

Plasmids—Human TIP150 cDNA was obtained from the Kazuza DNA Research Institute (Chiba, Japan) as described previously (14, 15). Briefly, to generate GFP-tagged TIP150, PCR-amplified cDNA was cloned into the pEGFP-C2 vector (Clontech, Mountain View, CA) with EcoRI and Sall digestion. TIP150 was then subcloned into the following vectors: p3×FLAG (Sigma) and pET-28a (Novagen). All TIP150 deletion mutants were created by PCR and confirmed by DNA sequencing. Murine CTN cDNA constructs (full-length CTN (amino acids 1–546)), N-terminal/terminal fragment(s), C-terminal/ fragment(s), Helix, hePro, SH3, ProLink, and LinkSH3 were fused with GST. The murine CTN non-phosphorylatable mutant (Y421F/Y466F/Y482F), phospho-mimicking mutant (Y421D/Y466D/Y482D), non-phosphorylatable mutant (Y497F/Y499F/Y541F), and phospho-mimicking mutant (Y497D/Y499D/ Y541D) were cloned into pGEX-6p-1 (GE Healthcare). GFP-tagged IQGAP1, N-WASP, and CTN full-length and/or truncations were generated by inserting PCR-amplified cDNAs into pEGFP-C2. All plasmid constructs were sequenced for verification.

To generate TAT-GFP-CT150-His fused proteins to perturb the TIP150-CTN interaction, an 11-amino acid TAT sequence followed by GFP and a His tag was inserted into the pET-22b vector. TAT-GFP-His fused proteins were expressed and purified as described previously (15).

siRNA and/or shRNA Transfection—The siRNA sequence used for silencing TIP150 was 5′-CAUCACGCAAAGUCG-GAGA-3′, described previously (14) (synthesized by Dharmacon Research, Inc., Boulder, CO), or SMARTpool (L-022219-00, Thermo Fisher Scientific). EB1 siRNA (5′-AAGUGAUAUU- CCAAGCUAAGC-3′) (39) and CTN siRNA (5′-CAAGCTT- CGAGGAAATGCTT-3′) were synthesized by Qiagen. As a control, a duplex-composing scrambled sequence was used (40, 41). The 21-mer oligonucleotide RNA duplexes were synthesized from Dharmacon Research, Inc. The shRNAs were constructed using the same targeting sequences as their siRNAs or as described previously (39). The vector was used as a control. All siRNAs and shRNAs were transfected into cells using Lipofectamine 2000 for 72 h, and the knockdown efficiency was confirmed by Western blotting and/ or immunofluorescence.

Pulldown and Immunoprecipitation Assay—Pulldown assays were carried out as described previously (42). Briefly, the GST-tagged proteins were purified from bacteria by glutathione-agarose chromatography. GST fusion protein-bound Sepharose beads were incubated with HEK293T cell lysates ectopically expressing GFP- or FLAG-tagged proteins or their deletion mutants, or with purified His-tagged proteins expressed in bacterial cells. After extensive washing, bound proteins were boiled in sample buffer, split into two aliquots, and separated by SDS-PAGE gradient gels (5–15%) manually prepared for Western blotting analysis. All pulldown assays were conducted at least three times.

FLAG-TIP150 and pEGFP-C2 vector or GFP-tagged proteins were co-transfected and expressed in HEK293T cells. The FLAG-tagged proteins were precipitated with anti-FLAG-M2
beads (Sigma). Beads were washed three times with lysis buffer, and proteins were separated on SDS-PAGE for transferring onto a nitrocellulose membrane for Western blotting using GFP antibody.

**Antibodies and Reagents**—Mouse serum against TIP150 was generated using HIS-TIP150-P (amino acids 801–980) from bacteria according to a standard protocol. Rabbit antibody against TIP150 was developed by YenZym (San Francisco, CA) as described previously (14). The following antibodies were obtained from commercial sources: anti-EB1 mouse antibody (BD Biosciences), anti-CTN rabbit antibody (Sigma), phospho-CTN (Tyr-421) antibody (Cell Signaling Technology), anti-GFP antibody (BD Biosciences), anti-FLAG antibody (M2, Sigma), and anti-tubulin antibody (DM1A, Sigma). FITC-conjugated secondary antibodies (Pierce) and rhodamine phalloidin (Invitrogen) were also obtained commercially.

**Immunofluorescence and Single Cell Migration**—Cells were seeded onto sterile, acid-treated, 12-mm coverslips in 24-well plates (Corning Glass Works) for transfection or drug treatment (43). Cells were fixed with cold methanol for 2 min at −20 °C. After washing three times with PBST (0.05% Tween 20 in PBS), cells were blocked with 1% bovine serum albumin (Sigma) for 45 min at room temperature. Cells were subsequently incubated with the indicated primary antibodies in a humidified chamber for 1 h at room temperature, followed by secondary antibodies for 1 h at room temperature. DNA was stained with DAPI (Sigma). Images were acquired with a DeltaVision wide-field deconvolution microscope (Applied Precision Inc.) as described previously (44). For single cell migration, MDA-MB-231 cells were cultured in a glass-bottom plate (Corning Glass Works) for transfection or drug treatment. For Scratch Assay—For the wound healing assay, confluent MDA-MB-231 cells transfected with the indicated shRNAs were scratched with a 10-μl pipette tip and then stimulated by EGF (100 ng/ml) or 20% serum at 37 °C for the indicated times. Images were collected with a 10 objective of an inverted microscope (Axiovert 200) coupled with an AxioCam-HS digital camera (Carl Zeiss). The relative healing velocities were measured using ImageJ software (National Institutes of Health).

**Total Internal Reflection Fluorescence Microscopic Analyses**—The microtubule plus end tracking assay was performed as described recently (20). The GTP analogue guanylyl-(α,β)-methylene-diphosphonate (GMPCPP) microtubule seeds were prepared by polymerizing 30 μM tubulin (at a bovine tubulin/rhodamine-tubulin/biotin-tubulin ratio of 30:2:1) in the presence of 1 mM GTP analogue guanylyl-(α,β)-methylene-diphosphonate (GMPCPP) (Jena Bioscience) at 37 °C for 40 min. The seeds were then centrifuged and resuspended in BRB80 buffer (80 mM K-PIPES (pH 6.8), 1 mM MgCl2, and 1 mM EGTA). These seeds were sheared with a 25-gauge needle before they were used to generate short seeds.

Flow chambers were prepared as described previously (20) and coated with 10% monoclonal anti-biotin antibody (Sigma), followed by blocking with 5% Pluronic F-127 (Sigma). After a brief wash, sheared microtubule seeds (125 nm) were added into the chamber. Tubulin polymerization mixture (30 μM tubulin in total containing 1:30 rhodamine-labeled bovine tubulin in BRB80, 50 mM KCl, 5 mM DTT, 1.25 mM magnesium GTP, 0.25 mg/ml k-casein, 0.15% methylcellulose (Sigma), an oxygen-scavenging system, and +TIPs) was introduced into the chamber to initiate polymerization. Unless stated otherwise, the final concentrations of +TIPs were 250 nm EB1 and 250 nm TIP150 or TIP150 plus CTN proteins. The temperature was kept at 25 °C. Images were collected with a superresolution microscope configured on an ELYRA system (Carl Zeiss). The laser intensities were kept at a low level to avoid photobleaching. For +TIPs tracking assays, 1 frame/s was taken. Plus end tracking of GFP-SKAP and its SXIP mutant was analyzed using kymographs in ZEN software (Carl Zeiss).

**Pyrene-labeled Actin Assembly**—To examine whether TIP150 alters actin assembly dynamics directly or indirectly via CTN, we adopted the pyrene-actin assembly assay reported previously (23). In short, pyrene-labeled actin was obtained from Cytoskeleton Inc. (catalog no. AP05-A) and used at a concentration of 5 μM. Because some polymerization of actin occurs during the freezing of samples, thawed actin was spun at 312,000 × g for 40 min before initiating assembly. Polymerization was initiated at the following final conditions: 5 mM Tris (pH 7.5), 0.5 mM ATP, 2 mM MgCl2, 10 mM KCl, and 0.2 mM DTT. Fluorescence was monitored continuously (excitation wavelength, 355 nm; emission wavelength, 407 nm) in the presence or absence of TIP150 and TIP150 plus CTN. Purified yeast cofilin was used as a control as reported previously (23).

**Data Analyses**—All fluorescence intensity measurements and statistical analyses were carried out using ImageJ software (National Institutes of Health) and GraphPad Prism. To determine the significant differences between means, unpaired Student’s t test assuming unequal variance was performed; differences were considered significant when p < 0.05.

**Author Contributions**—X. D., and X. L. conceived the project. G. A., J. Z., H. W., P. X., Z. W., F. M., and S. Z. designed and performed most biochemical experiments. J. Z., Y. L., J. J., X. Z., W. W., K. T., J. Q., F. O. A., P. H., and X. D. designed and performed cell biological characterization. G. A., J. Z., S. Z., and K. T. performed the in vitro reconstitution experiment and data analyses. G. A., J. Z., D. L. H., P. H., X. L., X. D., and X. Y. wrote the manuscript, and all authors read and approved the manuscript.

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