Supplementary Figure 1

Recovery of podosomes after nocodazole washout. (A) Time-course of podosome recovery. The time after nocodazole washout is indicated. Podosomes are visualized by actin labeling with GFP-β-actin. (B and C) quantification of the number of podosomes per cell (B) and the percentages of cells with more than 10 podosomes (C) in DMSO-treated (control), nocodazole-treated for 1 hour, and nocodazole treated cells incubated in the medium without nocodazole for 2 hours. Cells were fixed and stained with phalloidin and vinculin antibody. Not less than 200 cells were analyzed for each type of experimental conditions. The statistical difference (p-values) was assessed using two-tailed Student's *t*-test.

Supplementary Figure 2

KANK1 and KANK2 localizations and function in THP1 and HT1080 cells. (A) Localization of GFP-KANK1 to podosomes in THP1 cell. The podosomes are labeled by mCherry-vinculin (red, upper row), while the GFP-KANK1 distribution is shown in green (middle row). Merged images are shown in the bottom row. Note that GFP-KANK1 is located at the periphery of the podosomes partially overlapping with vinculin-positive rings. (B) Line scan profile through the individual podosome shown in the third column of (A). KANK1 is located to the outer ring surrounding the vinculin ring of podosome. (C and D) KANK2 is localized to podosomes in THP1 cell (C) but its knockdown did not result in podosome disruption (D). Podosomes were labeled by phalloidin staining of actin (red). KANK2 and microtubules were visualized by indirect immunofluorescence staining with antibodies against KANK2 (green) and α-tubulin (white), respectively. (E) Effects of knockdown of KANK1 and KANK2 on cell projected area (μm^2). KANK1 knockdown slightly decreases cell size while KANK2 knockdown markedly increased it. Each dot corresponds to individual cells. The statistical significance of the differences was assessed using two-tailed Student's *t*-test. (F) KANK1 knockdown HT1080 cell labeled with α-tubulin (green) and paxillin (red) antibodies. The cell preserved well-developed microtubule network (left) and form enlarged focal adhesions (right). The measurements of focal adhesion area is shown in Figure 1G. (G) Overexpression of talin-binding GFP-

KANK1-KN resulted in displacement of endogenous KANK2 from the focal adhesions. Two cells labeled with antibody against KANK2 (red) are shown. GFP-KANK1-KN (green) is expressed only in the upper one. Note that the lower cell which does not express GFP-KANK1-KN shows prominent KANK2-positive focal adhesions while the upper cell demonstrating GFP-KANK1-KN localization to focal adhesions show much lower level of endogenous KANK2 in focal adhesions.

Supplementary Figure 3

Effects of KANK1 and KANK2 constructs on podosomes and focal adhesions. (A) A map diagram depicting the domain structures of KANK1 and KANK2, and presenting their deletion mutants. KANK1 domain organization is based on data from ref (Bouchet et al., 2016). Putative EB1 binding site indicated according to the data for Drosophila KANK1 (Clohisey et al., 2014). KANK1 full-length and deletion mutant GFP fusion constructs were described in (Bouchet et al., 2016). KANK2 domain structures is based on data from ref (Sun et al., 2016). Putative binding domains for liprins and KIF21A are indicated by analogy with KANK1. The full-length KANK2 and its deletion mutant GFP fusion constructs were described in (Sun et al., 2016). (B-G) Effects of full-length KANK1 and its deletion mutants on the podosomes in THP1 cells. In each panel the image of podosomes visualized by actin-labeling with mCherry-UtrCH (red) is shown on the left, localization of corresponding GFP fusion constructs of KANK1 or its deletion mutants (green) is shown in the middle, and the merged image - on the right. (B and C) Rescue of podosomes in KANK1 knockdown cells (shown in Figure 1D right panel) by full-length GFP-KANK1 (B) and GFP-KANK1ΔANKR (C). (D and E) GFP-KANK1-KN (D) and GFP-KANK1-CC-Cter (E) did not rescue podosomes in KANK1-depleted cells, and their overexpression in cells transfected with control siRNA did not produce any morphological effect (F and G, respectively). See measurements of podosomes in Figure 1H and I. (H-M) Effects of full-length KANK2 and its deletion mutants on the focal adhesions in HT1080 cells. In each panel the focal adhesions were visualized by paxillin antibody staining (red, left image). The localization of GFP fusion constructs of KANK2 or its deletion

mutants (green) is shown in the middle image, and the merged image - on the right. (H and I) Full-length GFP-KANK2 (H) and GFP-KANK2 (1-670) (I) abolished the increase of focal adhesion size in KANK2 knockdown cell (shown in Figure 1D, left panel), while GFP-KANK2-KN (J) and GFP-KANK2ΔKN (K) did not. See measurements of focal adhesion size in Figure 1G. (L and M) The overexpression of neither GFP-KANK2 (1-670) (L) nor GFP-KANK2ΔKN (M) produced any effects in cells transfected with control siRNA.

Supplementary Figure 4

Myosin-IIA knockdown abolished effect of microtubules on focal adhesions in HT1080. (A) The microtubules were labeled with α-tubulin antibody staining (green, top panel) and focal adhesions in the same cells with antibody to paxillin (red, bottom panel). Untreated myosin-IIA knockdown cell is shown in the left, the similar cell treated with nocodazole in the middle and the cell after nocodazole washout on the right. Note that focal adhesion size in myosin-IIA knockdown cell is small and did not change upon microtubule disruption or outgrowth. The measurements of the focal adhesion size after corresponding treatments are shown in B. (B) The measurements of total focal adhesion area per cell in control and Myosin-IIA knockdown cells upon nocodazole treatment and washout. The cells were fixed and stained with antibodies to paxillin after respective treatments. Nz=nocodazole. Not less than 15 cells were assessed for each type of treatment. The statistical significance of the difference (p-values) was estimated by two-tailed Student's *t*-test, the range of P-values >0.05(non-significant), ≤ 0.05, ≤0.01, ≤0.001, ≤ 0.0001 are denoted by "ns", one, two, three and four asterisks (*), respectively.

(C) Myosin-IIA knockdown abolished the effect of KANK2 knockdown on focal adhesion size. The cells were labeled with non-muscle MHC-IIA antibody (green, upper panel) and with paxillin (red, bottom panel). Myosin-IIA knockdown cell is shown in the left pair of images, KANK2 knockdown cell in the middle, and cell with double myosin-IIA and KANK2 knockdown on the right. Notice small focal adhesion in myosin-IIA knockdown cell (left), augmented focal adhesions and myosin-II

filaments in KANK2 knockdown cell (middle) and reduction of focal adhesion size in cell with double myosin-IIA and KANK2 knockdown cell (right).

Supplementary Figure 5

Results of pull-down assay showing the RhoA-GTP level in KANK1 and KANK2 knockdown HT1080 and THP1 cells. Western blots of RhoA-GTP level and of total RhoA in the corresponding samples are shown in the upper snd lower rows, respectively. (A) RhoA-GTP levels in HT1080 (left) and THP1 (right) cells treated for 1 hour with DMSO (control), nocodazole (1μM) or 30 minutes (HT1080) or 2 hours (THP1) following the nocodazole washout. (B) Effects of KANK1 and KANK2 knockdowns on the RhoA-GTP level in HT1080 (left) and THP1 cells (right) as compared to the effects of nocodazole treatment. In HT1080 cells, the knockdown of either KANK1 or KANK2 led to increase of RhoA-GTP level similar to nocodazole-treatment. In THP1 cells, KANK1 knockdown increases the RhoA-GTP level in the same degree as nocodazole treatment, while KANK2 knockdown does not change the RhoA-GTP level as compared to control.

Supplementary Figure 6

GEF-H1 knockdown prevented the effect of microtubule disruption or KANK1 depletion on myosin-II filaments and podosomes in THP1 cells. (A) Control (left panel) and GEF-H1 knockdown (right panel) THP1 cells demonstrate intact podosomes visualized by the actin cores (red), peripheral rim of myosin-II filaments labeled by myosin-II heavy chain (green) and well-developed array of radial and circumferential microtubules (white). (B) Nocodazole treatment of control (left panel) and GEF-H1 knockdown (right panel) THP1 cells. The labeling of actin, myosin-II heavy chain and microtubules is the same as in (A). Note that nocodazole treatment disrupted podosomes and enhance the amount of myosin-II filaments in control but not in GEF-H1 knockdown cell while disruption of microtubules was complete in both cases. (C) KANK1 knockdown THP1 cell (left panel) and cell with double knockdown of GEF-H1 and KANK1. Labeling of actin, myosin-II filaments and

microtubules is the same as in (A) and (B). Note that KANK1 knockdown led to disassembly of podosomes and appearance of actin filament bundles, as well as increase in the amount of myosin-II filaments. The cell lacking both GEF-H1 and KANK1 demonstrate intact podosomes and low level of myosin-II filaments. The microtubules are well preserved in both cases.

Supplementary Figure 7

(A-C) Focal adhesions (paxillin, red) in HT1080 cells from control (A, left panel) and GEF-H1 knockdown (A, right panel) cultures. Note slight increase in focal adhesion size in GEF-H1 knockdown cell. (B) Effect of nocodazole treatment for 1 hour on control cell (left panel) and GEF-H1 knockdown cell (right panel). (C) Cells transfected with KANK2 siRNA (left panel) or with KANK2 and GEF-H1 siRNAs together (right panel). Note that both nocodazole treatment and KANK2 knockdown increased the size of focal adhesions in control but not in GEF-H1 knockdown cells. Scale bars, 10 μm. (D and E) GEF-H1 knockdown prevented increase in focal adhesion area induced by expression of GFP-KANK2-KN. (D) Cell transfected with control siRNA and GFP-KANK2-KN (green) demonstrates large and numerous focal adhesions (red). (E) The cell transfected with GEF-H1 siRNA and GFP-KANK2-KN demonstrates few focal adhesions of small size, similarly to cells transfected with GEF-H1 siRNA alone (A, right panel). (F) Quantification of the total focal adhesion area per cell in conditions corresponding to that shown in (A-E). (G) Western blot demonstrating siRNA-mediated depletion of GEF-H1, KANK2, and both of them in HT1080 cells. α-tubulin is used as loading control. (H) The sequence of showing the image of the GEF-H1 knockdown cell labeled for microtubules (upper panels), myosin-II filaments (middle panels) and focal adhesions (bottom panels) in the course of microtubule outgrowth after nocodazole washout. The microtubules, myosin-II filaments and focal adhesions were labeled with mApple-MAP4, GFP-MRLC and mTFP-vinculin, respectively. See also Movie S14. Unlike the disruptive effect of microtubule outgrowth in control cells on myosin-II filaments and focal adhesions (Figure 3A and B), the recovery of microtubules in GEF-H1 knockdown

cell was accompanied by only partial disruption of myosin-II filaments and focal adhesions (see quantification in I). (J) The measurements of total focal adhesion area per cell in control and GEF-H1 knockdown cells upon nocodazole treatment and washout. The cells were fixed and stained with antibodies to paxillin after respective treatments. Nz=nocodazole. Not less than 45 cells were assessed for each type of treatment. The statistical significance of the difference (p-values) was estimated by two-tailed Student's *t*-test, the range of P-values >0.05(non-significant), ≤ 0.05, ≤0.01, ≤0.001, ≤ 0.0001 are denoted by "ns", one, two, three and four asterisks (*), respectively.

Movie legends

Supplementary movie 1
Assembly of new podosomes labeled by GFP-β-actin in THP1 cell after nocodazole washout with complete medium. The movie images were recorded at 2-minute intervals over a period of 60 minutes using SIM. Display rate is 10 frames/sec. The movie corresponds to the time-lapse series shown in Supplementary Figure 1A. Scale bar, 5 μm.

Supplementary movie 2
Disruption of podosomes labeled by RFP-lifeact (red, left) in THP1 cell treated with 1 μM nocodazole was accompanied by a massive burst of myosin-II filament assembly that aligned in stacks, as visualized by GFP-MRLC (green, right) using SIM. Single plane close to the substrate is shown. The frames were recorded at 15-second intervals over a period of 30 minutes using SIM. Display rate is 10 frames/sec. The movie corresponds to the time-lapse series shown in Figure 2A. Scale bar, 5 μm.

Supplementary movie 3
Myosin-II filament formation accompanies focal adhesion augmentation in HT1080 cell treated with 1 μM nocodazole. Focal adhesions are labeled with mApple-paxillin (red), myosin-II filaments are labeled with GFP-MRLC (green) and imaged using SIM. The imaging started immediately after nocodazole addition. Single plane close to the substrate is shown. The frames were recorded at 1-minute intervals over a period of 30 minutes. Display rate is 10 frames/sec. The movie corresponds to the time-lapse series shown in Figure 2B. Scale bar, 10 μm.

Supplementary movie 4
Microtubule outgrowth induces disassembly of myosin-II filaments in HT1080 cell. The imaging started immediately after nocodazole washout. Microtubules are labeled with mApple-MAP4 (left) and myosin II filaments are labeled with GFP-MRLC (right) and imaged using SIM. For microtubules, z-projection of all focal planes is shown; for myosin-II filaments, single plane near the substrate is shown. The frames were recorded at 15 seconds intervals over a period of 10 minutes. Display rate is 10 frames/sec. The movie corresponds to the time-lapse series shown in Figure 3A. Scale bar, 10 μm.

Supplementary movie 5
Changes in traction forces after microtubule outgrowth in HT1080 cell. The imaging started immediately after nocodazole washout. Traction stresses (left) computed as

described in Materials and Method section are presented in spectrum scale (Pa); the scale is shown on the farmost left. Focal adhesions are labeled with RFP-Zyxin (right) and imaged using spinning disk confocal microscopy. Single plane close to the substrate is shown. The frames were recorded at 1-minute intervals over a period of 31 minutes. Display rate is 10 frames/sec. The movie corresponds to the time-lapse series shown in Figure 3C. Scale bar, 10 μm.

Supplementary movie 6
KANK2 knockdown suppresses disassembly of myosin-II filaments induced by microtubule outgrowth in HT1080 cell. The imaging started immediately after nocodazole washout. Microtubules are labeled with mApple-MAP4 (left), myosin II filaments are labeled with GFP-MRLC (right) and imaged using SIM. For microtubules, z-projection of all focal planes is shown; for myosin-II filaments, single plane near the substrate is shown. The frames were recorded at 15-second intervals over a period of 5 minutes. Display rate is 10 frames/sec. The movie corresponds to the time-lapse series shown in Figure 3E. Scale bar, 10 μm.

Supplementary movie 7
Disruption of podosomes labeled by RFP-lifeact (red, left) in THP1 cell treated with 1 μg/ml CN03 was accompanied by an increased assembly of myosin-II filaments visualized by GFP-MRLC (green, right), similar to nocodazole-treated cell. Single plane close to the substrate is shown. The frames were recorded at 1-minute intervals over a period of 40 minutes using SIM. Display rate is 10 frames/sec. The movie corresponds to the time-lapse series shown in Figure 4A. Scale bar, 5 μm.

Supplementary movie 8
Dynamics of podosomes labeled by RFP-lifeact (red) in THP1 cell treated with 0.2% DMSO. Note that myosin-II filaments visualized by GFP-MRLC (green) are localized to the peripheral rim of the cell and are absent at sites of podosome assemblies. Single plane close to the substrate is shown. The frames were recorded at 10-seconds intervals over a period of 30 minutes using SIM. Display rate is 10 frames/sec. The movie corresponds to the time-lapse series shown in Figure 4B.

Supplementary movie 9
Inhibition of Rho kinase by addition of 30 μM Y-27632 induced disassembly of myosin-II filaments visualized by GFP-MRLC (green) but did not affect podosome dynamics in THP1 cell. Podosomes are labeled by RFP-lifeact (red) and imaged

using SIM. Single plane close to the substrate is shown. The frames were recorded at 10-seconds intervals over a period of 30 minutes. Display rate is 10 frames/sec. The movie corresponds to the time-lapse series shown in Figure 4C. Scale bar, 5 μm.

Supplementary movie 10

Inhibition of Rho kinase by 100 μM Y-27632 prevented the nocodazole-induced burst of myosin-II filaments and disruption of podosomes in THP1 cell. The imaging started at the 30th minute after addition of Y-27632 and was continued for another hour in the presence of both Y-27632 and 1 μM nocodazole. Single plane close to the substrate is shown. The frames were recorded at 2-minute intervals over a period of 40 minutes. Podosomes and myosin II filaments are labeled with RFP-lifeact (red) and GFP-MRLC (green), respectively, and imaged by SIM. The movie corresponds to the time-lapse series shown in Figure 4D. Scale bar, 5 μm.

Supplementary movie 11

Disassembly of myosin-II filaments by 30 μM Y-27632 results in recovery of podosomes in THP1 cell pre-treated with nocodazole for 30 minutes as visualized by SIM. Podosomes and myosin II filaments are labeled with RFP-lifeact (red) and GFP-MRLC (green), respectively, and imaged by SIM. The imaging started at the 30th minute after addition of nocodazole and was continued for another hour in the presence of both Y-27632 and 1 μM nocodazole. Single plane close to the substrate is shown. The frames were recorded at 2-minute intervals over a period of 40 minutes. Display rate is 10 frames/sec. The movie corresponds to the time-lapse series shown in Figure 4E. Scale bar, 5 μm.

Supplementary movie 12

Inhibition of Rho kinase by treatment with 30 μM Y-27632 in KANK1-depleted THP1 cell induced the formation of *de novo* podosomes, approximately 30 minutes after addition of the drug. Podosomes and myosin-II filaments are labeled with RFP-lifeact (red) and GFP-MRLC (green), respectively, and imaged by SIM. Single plane close to the substrate is shown. The frames were recorded at 30-second intervals over a period of 60 minutes using SIM. Display rate is 10 frames/sec. The movie corresponds to the time-lapse series shown in Figure 4F. Scale bar, 5 μm.

Supplementary movie 13

Constitutively active RhoA and ROCK prevented the disruption of focal adhesions upon microtubule outgrowth in HT1080 cells. Control cell (left) and cells expressing

constitutive active form of RhoA (middle) or ROCK (right) are imaged just after nocodazole washout by TIRF microscopy. Focal adhesions are labeled with mApple-paxillin. The frames were recorded at 30-second intervals over a period of 30 minutes. Display rate is 10 frames/sec. The movie corresponds to the time-lapse series shown in Figure 4I. Scale bar, 10 μm.

Supplementary movie 14

GEF-H1 knockdown suppresses disassembly of myosin-II filaments and focal adhesions induced by microtubule outgrowth in HT1080. The imaging started immediately after nocodazole washout. Microtubules are labeled with mApple-MAP4 (left), myosin II filaments are labeled with GFP-RLC (right); focal adhesions are labeled with mTFP-vinculin (right), and were imaged using SIM. For microtubules, z-projection of all focal planes is shown; for myosin-II filaments and focal adhesions, single planes near the substrate are shown. The frames were recorded at 15 seconds intervals over a period of 10 minutes. Display rate is 10 frames/sec. The movie corresponds to the time-lapse series shown in Supplementary Figure 7H. Scale bar, 10 μm.