Invadopodia are integrin-mediated adhesions with abundant PI(3,4)P₂. However, the functional role of PI(3,4)P₂ in adhesion signaling remains unclear. Here, we find that the PI(3,4)P₂ biogenesis regulates the integrin endocytosis at invadopodia. PI(3,4)P₂ is locally produced by PI3KCA and SHIP2 and is concentrated at the trailing edge of the invadopodium arc. The PI(3,4)P₂-rich compartment locally forms small puncta (membrane buds) in a SNX9-dependent manner, recruits dynein activator Hook1 through AKTIP, and rearranges into micrometer-long tubular invaginations (membrane tubes). The uncurving membrane tube extends rapidly, follows the retrograde movement of dynein along microtubule tracks, and disconnects from the plasma membrane. Activated integrin-beta3 is locally internalized through the pathway of PI(3,4)P₂-mediated membrane invagination and is then actively recycled. Blockages of PI3K, SHIP2, and SNX9 suppress integrin-beta3 endocytosis, delay adhesion turnover, and impede transwell invasion of MEF-Src and MDA-MB-231 cells. Thus, the production of PI(3,4)P₂ promotes invasive cell migration by stimulating the trafficking of integrin receptor at the invadopodium.

The conversions between phosphoinositide lipids may involve multiple steps and are dynamically regulated by site-specific kinases and phosphatases. In particular, the production of PI(3,4)P₂ can be controlled by two different pathways. Diphosphorylation of PI(3,4,5)P₃ by phosphoinositide 5-phosphatases can result in the production of PI(3,4)P₂ (17). On the other hand, class II PI3Ks can directly phosphorylate PI(4)P and contribute to PI(3,4)P₂ production (18).

In the invadopodium, PI(3,4,5)P₃ and PI(3,4)P₂ act as important upstream messengers to initiate F-actin polymerization (3). However, the functional role of phosphoinositide lipids in adhesion turnover and integrin trafficking at invadopodia remains unclear. Here, we use the invadopodium model system of Src-transformed fibroblast (MEF-Src) (3, 19, 20) and MDA-MB-231 breast cancer cell to reveal how the PI(3,4)P₂ production and the microtubule-mediated retrograde transport trigger the membrane tubulation, stimulate the integrin endocytosis, and promote the invasive cell migration.

**Results**

**Asymmetrical Distribution of PI(3,4)P₂ across the Invadopodium Arc.** Species-specific binding probes have been used to report the dynamic distribution of phosphoinositide lipids inside the cell (21). While the previous study and ours have reported the enrichment of PI(3,4,5)P₃ and PI(3,4)P₂ at the invadopodium using the model system of MEF-Src cells (SI Appendix, Fig. S1A) (19, 22), the distributions of these two lipids within the invadopodium were rather different. PI(3,4)P₂, visualized by the PH-Tapp1

**Significance**

The dynamic distribution of phosphoinositide lipids orchestrates intracellular compartmentalization and protein sorting. Here, we report the PI(3,4)P₂-dependent endocytosis of adhesion receptor integrin-beta3 at the invadopodium and its implication in promoting invasive cell migration. PI(3,4)P₂-rich compartment in the plasma membrane contains integrin-beta3 and is actively internalized through SNX9-mediated membrane invagination as well as dynein-mediated membrane tubulation along cortical microtubule tracks. Accelerated integrin endocytosis promotes adhesion turnover, and subsequent integrin exocytosis further supports cell migration. In general, the functional link between PI(3,4)P₂ biogenesis and membrane internalization can be applicable to other membrane molecules at the invadopodium and provide insights into the regulation of cell migration.

**Author contributions:** Z.F. and C.-h.Y. designed research, performed research, contributed new reagents/analytic tools, analyzed data, and wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2017645118/-/DCSupplemental.

Published May 4, 2021.

**PNAS 2021 Vol. 118 No. 19 e2017645118**

https://doi.org/10.1073/pnas.2017645118 | 1 of 12
probe, was asymmetrically accumulated at the trailing edge of the invadopodium arc, compared to the plasma membrane marker CAAX (Fig. 1A and D). On the other hand, the distribution of PI(3,4,5)P₃, revealed by the PH-BTK probe, was homogeneously enriched across the invadopodium arc (Fig. 1B and D). PI(4,5)P₂, PI(4)P, and PI(3)P, reported by PH-PLCd, P4M-SidM, and PX-p40phox probes respectively, were not distinctly enriched at the invadopodium arc (Fig. 1C and D and SI Appendix, Fig. S1B–E). To determine the kinase involved in the local production of PI(3,4)P₂ (Fig. 1E), we examined the subcellular localization of various PI3K catalytic subunits. Green fluorescent protein (GFP)-tagged class I PIK3CA and PIK3CB were located at the invadopodium, while PIK3CG and PIK3CD appeared to be diffusive in the cytoplasm (SI Appendix, Fig. S1F). Analyses using RT-qPCR suggested that PIK3CA, rather than PIK3CB, was the major catalytic subunit expressed in MEF-Src (SI Appendix, Fig. S1G). Class II PI3Ks, including PIK3C2A, PIK3C2B, and PIK3C2G, were not enriched at invadopodia (SI Appendix, Fig. S1H).

Fig. 1. PI(3,4)P₂ concentrates at the trailing edge of the invadopodium arc. (A) GFP-PH-Tapp1 (the PI(3,4)P₂ probe) is enriched at the trailing edge of the invadopodium arc in MEF-Src in comparison with the plasma membrane marker mCherry-CAAX. (B) PH-BTK-GFP (the PI(3,4,5)P₃ probe) is homogeneously enriched within the invadopodium arc. (C) PH-PLCd-GFP (the PI(4,5)P₂ probe) is not distinctly enriched in the invadopodium arc. (D) The intensity profiles of PH-Tapp1, PH-BTK, PH-PLCd, CAAX, and F-actin across the invadopodium arc (outside-in line scan; n = 21, 38, 38, 89, and 78, respectively). (E) The pathway involved in the biogenesis of PI(3,4)P₂. (F) Endogenous PIK3CA is located at the invadopodium. (G) The intensity profiles of PIK3CA and F-actin across the invadopodium arc (outside-in line scan, n = 21). (H) The Western blot indicates the knockdown of PIK3CA by the shRNA. (I) The schematic diagram of ratiometric enrichment analysis. (J) The knockdown of PIK3CA reduces the FOI of mCherry-PH-Tapp1 at the invadopodium. Reintroducing PIK3CA rescues the FOI of PH-Tapp1. (K) The FOI of PH-Tapp1 after PIK3CA knockdown and rescue as indicated. The statistical information is in SI Appendix, Table S1A. (L) Endogenous SHIP2 is located at the invadopodium and concentrates at the trailing edge of the invadopodium arc. (M) The intensity profiles of SHIP2 and F-actin across the invadopodium arc (outside-in line scan, n = 23). (N) The Western blot indicates the knockdown of SHIP2 by the shRNA. (O) The knockdown of SHIP2 reduces the FOI of mCherry-PH-Tapp1 at the invadopodium. Reintroducing SHIP2 rescues the FOI of PH-Tapp1 at the invadopodium. (P) The FOI of PH-Tapp1 after SHIP2 knockdown and rescue as indicated. The statistical information is in SI Appendix, Table S1B. The invadopodium arc is labeled by F-actin marker EBFP2-UtrCH. All experiments have been independently repeated three times. The graphs show mean ± SEM. One-way ANOVA with Dunnett’s test is used for the statistical analysis. Ns, P > 0.1234; **P < 0.0021 (Scale bars, 10 μm).
Knockdown of PIK3CA Suppresses the Production of PI(3,4,5)P3. Endogenous PIK3CA concentrated at the invadopodium (Fig. 1F). In addition, PIK3CA showed moderate enrichment at the trailing edge of invadopodium arc (Fig. 1G). We next investigated whether loss of PIK3CA affects the PI(3,4,5)P3 production. The knockdown of PIK3CA expression was achieved by short hairpin RNA (shRNA) (Fig. 1H). The impact of PIK3CA knockdown on the phosphoinositide production was quantified by the confocal microscopy and the ratiometric image analysis (Materials and Methods). The fold of increase (FOI) of the phosphoinositide binding probe inside a three-dimensional volume of the invadopodium (3 μm in height) was determined (Fig. 1K). While the formation of the invadopodium arc was not disrupted by the PIK3CA knockdown, the FOIs of PH-Tapp1 and PH-BTK were the significantly decreased in comparison with the control (Fig. 1J and K and SI Appendix, Fig. S1 I and J). Reintroducing the resistant form of PIK3CA restored the enrichment of PH-Tapp1 and PH-BTK in the knockdown cells. The treatment with PIK3CA inhibitor A66 (100 nM) also resulted in similar decreases in the FOI of PH-Tapp1 and PH-BTK in comparison with the dimethyl sulfoxide (DMSO) control (SI Appendix, Fig. S1 K–N) (23). It appears that PIK3CA is the key kinase related to the production of PI(3,4,5)P3 and PI(3,4)P2 at the invadopodium.

SHIP2 Is the Major Phosphoinositide 5-Phosphatase to Produce PI(3,4)P2 at the Invadopodium. As PI(3,4,5)P3 is the leading source of PI(3,4)P2 production on the plasma membrane (24), we sought to determine the major phosphoinositide 5-phosphatase involved (Fig. 1E). Among the nine phosphatases examined, SHIP2, INPP5E, SYNJ2, SHIP1, and INPP5J were enriched at invadopodia, while INPP5K, SYNJ1, INPP5B, and OCRL1 were not (SI Appendix, Fig. S2A). In particular, the endogenous expression of SHIP2 (also known as INPPL1) was high, and ectopically introduced SHIP2 greatly promoted the FOI of PH-Tapp1 (SI Appendix, Fig. S2 A and B). Endogenous SHIP2 was located at the trailing edge of the invadopodium arc (Fig. 1L). The asymmetrical distribution of SHIP2 remarkably resembled that of PH-Tapp1 (Fig. 1M). When SHIP2 was knocked down by the shRNA (Fig. 1N), the formation of the invadopodium arc in MEF-Src was not disrupted. However, the FOI of PH-Tapp1 in the invadopodium dropped significantly in comparison with the control (Fig. 1 O and P). At the same time, the FOI of PH-BTK in the invadopodium rose (SI Appendix, Fig. S2 C and D). Reintroducing the resistant form of SHIP2 in the knockdown cells reversed the changes of PH-Tapp1 and PH-BTK, respectively. Likewise, when the SHIP2 phosphatase activity was inhibited by AS1949490 (10 μM) (25), the lower FOI of PH-Tapp1 and the higher FOI of PH-BTK were also observed compared to the DMSO control (SI Appendix, Fig. S2 E–H). These data suggest that SHIP2 is the major phosphoinositide 5-phosphatase which contributes to the production and the asymmetrical distribution of PI(3,4,5)P3 and PI(3,4)P2 at the invadopodium arc.

PI(3,4)P2-Rich Compartment Actively Develops into Invaginated Membrane Tubes and Endocytic Vesicles. The local production of PI(3,4)P2 has been reported in various endocytosis events (26, 27). At the trailing edge of the invadopodium arc, we found that the PI(3,4)P2 reporter PH-Tapp1 was further enriched at the invaginated small puncta (membrane buds) (Fig. 2 A and B and Movie S1). The PI(3,4,5)P3 probe PH-BTK and clathrin light chain were not observed at the membrane bud (SI Appendix, Fig. S3 A and B). The enrichment of PI(3,4)P2 at the membrane bud was also confirmed by the specific recruitment of other PI(3,4)P2 reporters, including PX-Tks5 and INPP4A (SI Appendix, Fig. S3 C–F). Over time, the invaginated membrane buds broke away from the plasma membrane. Intriguingly, a population of the membrane buds extended inwardly and then formed micrometer-long straight membrane tubulations (membrane tubes) (Fig. 2B).

SNX9 Is a PI(3,4)P2 Effector and Promotes the Membrane Invagination. BAR-domain proteins are known to sense and to generate the membrane curvature. Specifically, we found that PX-BAR–domain protein SNX9 was enriched at PH-Tapp1–positive membrane buds and colocalized with DNM2, the key protein involved in membrane fission (Fig. 4 A and F). Other BAR-domain proteins, such as...
Fig. 2. PI(3,4)P2-rich compartment forms the invaginated membrane bud and tube along cortical microtubule tracks. (A) Time-projected images of GFP-PH-Tapp1 and F-actin in MEF-Src (40 to 300 s, color coded). Over time, there are numerous PH-Tapp1-positive membrane invaginations behind the invadopodium arc. (B) Time-lapse images of the boxed region in A (6 x 8 μm²). PH-Tapp1 puncta (membrane buds, arrowheads) initially appear at the trailing edge of the invadopodium arc, elongate inwardly, and become straight tubular membrane structures (membrane tubes). See Movie S1. (C) The histogram of the membrane tube elongation rate (n = 43). (D) Membrane marker GPI-mCherry colocalizes with GFP-PH-Tapp1 in the membrane tube. See Movie S2. (E) Time-lapse images of the boxed region of D (1 x 6 μm²; the arrow indicates the base of the tube). Membrane tubes are repeatedly formed and then detached from the same location behind the invadopodium arc (arrowheads). (F) The intensity profiles of PH-Tapp1 and GPI at t₁ (the fully grown tube) and t₂ (the detached tube) in E. (G) Early endosome marker GFP-Rab5 accumulates in the vesicular compartment behind the mCherry-PH-Tapp1-labeled invadopodium arc. See Movie S3. (H) The kymograph along the boxed region of G (the arrow indicates the base of the tube). Rab5 dynamically associates with the detached membrane compartment (arrowheads). (I) The temporal intensity changes of PH-Tapp1 and Rab5 in the boxed region of H. (J) The GFP-PH-Tapp1-positive membrane bud elongates, breaks away from the invadopodium arc and moves along EMTB-mCherry-labeled microtubule track (arrowheads). The rectangular Inset shows the time-lapse images of the boxed region (6 x 18 μm²). See Movie S4. (K) After 15 min of the nocodazole treatment (10 μM), the formation of the GFP-PH-Tapp1-positive membrane tubes is greatly suppressed compared to the DMSO control. (L) The number of membrane tubes within the 15 min before and after the treatment as indicated (n = 16 and 15). The statistical information is in SI Appendix, Table S1C. The invadopodium arc is labeled by F-actin marker LifeAct-mRuby (in A and B) or EBFP2-UtrCH (in D, E, G, H, J, and K). All experiments have been independently repeated three times. Unpaired two-tailed Student’s t test is used for the statistical analysis. Ns, P > 0.1234; ***P < 0.0002 (Scale bars, 5 μm).
Fig. 3. Dynein and AKTIP/Hook1 complex regulate the formation of the PI(3,4)P2-rich membrane tube. (A) mCherry-Arp1 is located at the tip of the GFP-PH-Tapp1-positive membrane tube. See Movie S5. (B) Time-lapse images of the boxed region in A (1 × 6 μm²; the arrow indicates the base of the tube). (C) The intensity profiles of PH-Tapp1 and Arp1 at t₀ (the initial tube formation) and t₁ (the fully-grown tube) in B. (D) mCherry-AKTIP colocalizes with Hook1-GFP at the EBFP2-PH-Tapp1-positive membrane bud. (E) The intensity profiles of PH-Tapp1, AKTIP, and Hook1 along the arrowhead in the inset of D (outside-in line scan, n = 11). (F) GFP-AKTIP colocalizes along the mCherry-PH-Tapp1-positive membrane tube. See Movie S6. (G) Time-lapse images of the boxed region in F (1.5 × 3.3 μm²; the arrow indicates the base of the tube). (H) The intensity profiles of PH-Tapp1 and AKTIP at t₀ (the initial tube formation) and t₁ (the fully-grown tube) in G. (I) The Western blot indicates the knockdown of AKTIP by the shRNA. (J) Hook1-GFP becomes absent from the mCherry-PH-Tapp1-positive membrane bud when AKTIP is knocked down. (K) Knockdown of AKTIP results in the accumulation of stationary mCherry-PH-Tapp1-positive vesicles behind the invadopodium arc (arrowhead in the inset). Reintroducing AKTIP, rather than putative membrane-binding mutant AKTIP-2RA, overturns the vesicle accumulation. (L) The number of PH-Tapp1-positive stationary vesicles (larger than 500 nm) per invadopodium arc is indicated. The statistical information is in SI Appendix, Table S1D. The invadopodium arc is labeled by F-actin marker EBFP2-UtrCH. All experiments have been independently repeated three times. The graphs show mean ± SEM. One-way ANOVA with Dunnett’s test is used for the statistical analysis. Ns, P > 0.1234; *P < 0.0332; ****P < 0.0001 (Scale bars, 5 μm).
endophilin A1 (N-BAR), AMPH1 (N-BAR), CIP4 (F-BAR), and IRSp53 (I-BAR) did not colocalize with PH-Tapp1–positive membrane buds (SI Appendix, Fig. S4 A–D). The overexpression of SNX9 was known to spontaneously trigger membrane tubulations (33). Therefore, SNX9 was weakly expressed in order to examine its spatiotemporal role in the biogenesis of the PI(3,4)P2-rich membrane tube. We found that the lipid-binding PX-BAR domain of SNX9 (201 to 595), rather than the SH3 domain (1 to 60), was sufficient to be recruited to membrane buds (SI Appendix, Fig. S4 E–J). During the membrane tube extension, full-length SNX9 and DNM2 stayed at the base of the membrane tube and subsequently dissociated as the membrane tube disconnected from the invadopodium (Fig. 4 B and C, between \( t_1 \) and \( t_2 \), and Movie S7).

We next investigated whether the recruitment of SNX9 depends on PI(3,4)P2. When the PI(3,4)P2 production was blocked by SHIP2 knockdown, the FOI of SNX9 at the invadopodium was suppressed (Fig. 4 D and E). Reintroducing the resistant form of SHIP2 recovered the recruitment of SNX9 in the knockdown cells. Likewise, we asked whether the formation of invaginated membrane buds is regulated by SNX9. We found that the knockdown of SNX9 greatly suppressed the number of PH-Tapp1–positive membrane buds, while the formation of the invadopodium arc was not disrupted (Fig. 4 F–H). Reintroducing the resistant form of SNX9 reversed the inhibitory effect in the knockdown cells. The direct association between the SH3 domain of SNX9 and the PRD domain of DNM2 was reported previously (34). Therefore, we asked whether SNX9 also regulates the recruitment of DNM2 to the PI(3,4)P2-rich compartment. DNM2 without PRD domain (1 to 742) was not enriched at the membrane bud and exhibited a diffusive pattern within the

![image](https://doi.org/10.1073/pnas.2017645118)
cell (SI Appendix, Fig. S4K). The recruitment of full-length DN2 to the trailing edge of the invadopodium was also suppressed when SNX9 was knocked down (SI Appendix, Fig. S4 L and M). Thus, SNX9 is the effector of PI(3,4)P₂ and regulates the membrane invagination and DN2M2 recruitment at the trailing edge of the invadopodium.

Invadopodia Stimulate the Trafficking of Integrin-beta3 Receptor. Adhesion receptors, such as integrins, play essential roles in the assembly of invadopodia. We found that integrin-beta3 concentrated at the PI(3,4,5)P₃-rich plasma membrane invagination when MEF-Src adhered on the fibronectin-coated substrate (Fig. 5A). We asked whether the formation of invadopodium promotes integrin-beta3 endocytosis. As endocytosed integrin-beta3 from the plasma membrane can retain the ligand-activated conformation (5, 9), we used the conformation-specific antibody LIB2S, which recognized the exposed ectodomain of activated integrin-beta3, to examine the integrin-beta3 endocytosis. Indeed, the level of endocytosed integrin-beta3 in the cytoplasm of invadopodium-positive MEF-Src was distinctly higher than that in wild-type MEF (Fig. 5B and C). We next investigated whether the exocytosis level of integrin-beta3 also increases. The pulse-chase assay was used to evaluate the exocytosis of integrin-beta3 (Materials and Methods). The conformation-specific antibody LIB2S was used to prelabel integrin-beta3 on the cell surface and was subsequently internalized. After the serum stimulation, the exocytosis level of LIB2S-labeled integrin-beta3 in MEF-Src was indeed higher than that in wild-type MEF (Fig. 5D and E). It appears that the invadopodium formation positively regulates the endocytosis and recycling of integrin-beta3.

PI(3,4)P₂-Mediated Membrane Invagination Promotes the Internalization of Integrin-beta3 and the Adhesion Turnover. As integrin-beta3 receptors were incorporated in the invaginated membrane tube, we sought to determine the role of PI(3,4)P₂ biogenesis in integrin-beta3 endocytosis. We found that internalized integrin-beta3 colocalized with autophosphorylated FAK (pY397-FAK) in the endosomal compartment (Fig. 5F and G). The endosomal pY397-FAK level can be examined by Western blot and used as a surrogate of integrin endocytosis when the other contribution of pY397-FAK from the cell–matrix adhesion site was quenched by cell detachment (Fig. 5F) (5, 9). Indeed, the higher endosomal level of pY397-FAK was observed in MEF-Src, rather than in MEF (Fig. 5F), and confirmed the higher level of endocytosed integrin-beta3 (Fig. 5B and C). The treatment with endocytosis inhibitor dynasore (20 μM) (35), as the negative control, eliminated the endosomal pY397-FAK level in the detached MEF-Src but did not alter the overall pY397-FAK level in the attached MEF-Src (Fig. 5I and SI Appendix, Fig. S5A). When the PI(3,4)P₂-mediated membrane invagination was blocked by PIK3CA, SHIP2, and SNX9 knockdown, the endosomal pY397-FAK level was significantly reduced compared to the scramble control (Fig. 5 J–L). Reintroducing PIK3CA, SHIP2, or SNX9 in the respective knockdown condition restored the endosomal pY397-FAK level (SI Appendix, Fig. S5B). The inhibition with A66 (100 nM) and AS1949490 (10 μM) also suppressed the pY397-FAK level in the detached MEF-Src, rather than that in the attached MEF-Src (SI Appendix, Fig. S5 A, C, and D). In agreement with the observation of endocytic vesicle accumulation, the endosomal pY397-FAK level remained elevated when AKTIP was knocked down (SI Appendix, Fig. S5E). Thus, PI(3,4)P₂-mediated membrane invagination, rather than dynemin-mediated membrane tubulation, is essential to promote the endosomal pY397-FAK level and integrin endocytosis.

As up-regulated integrin endocytosis can promote the turnover of the preexisting adhesion structure, we next utilized photoconvertible mEOS2-tagged integrin-beta3 to examine the functional impact of PI(3,4)P₂-mediated integrin-beta3 endocytosis in the adhesion stability. After a single photoactivation by the total internal reflection fluorescence (TIRF) microscopy, the intensity changes of photoconverted integrin-beta3-mEOS2 at the invadopodium were monitored over time. We found that the intensity declines of photoconverted integrin-beta3-mEOS2 in MEF-Src were significantly slower in PIK3CA, SHIP2, and SNX9 knockdown MEF-Src compared to the scramble control (Fig. 5 M and N). In agreement with the observation of elevated endosomal pY397-FAK level, both AKTIP knockdown and control MEF-Src exhibited fast intensity declines of photoconverted integrin-beta3-mEOS2 (SI Appendix, Fig. S5 F and G). Altogether, these data indicate that the PI(3,4)P₂ production and the SNX9-mediated membrane invagination positively stimulate the integrin-beta3 endocytosis and promote the adhesion turnover.

Suppression of PI(3,4)P₂-Mediated Integrin Endocytosis Impedes the Invasive Migration of MEF-Src and MDA-MB-231 Cells. Invadopodium-forming cells, such as MEF-Src and MDA-MB-231 cell, can actively penetrate and migrate through the matrix barrier. We asked whether the invasive cell migration depends on the PI(3,4)P₂-mediated integrin endocytosis. With the fetal bovine serum (FBS) as the chemoattractant, MEF-Src, rather than nontransformed MEF, showed the greater ability to migrate through a Matrigel-coated transwell chamber (Fig. 6A and B). The knockdown of PIK3CA, SHIP2, and SNX9, which suppressed the PI(3,4)P₂-mediated integrin endocytosis, effectively impeded the transwell migration of MEF-Src (Fig. 6 C and D). Reintroducing PIK3CA, SHIP2, or SNX9 in the respective knockdown condition restored the invasive capacity. On the other hand, MEF-Src with AKTIP knockdown increasingly suffered from cell death (30), and their transwell migrations were not quantified. Likewise, MDA-MB-231 cells assemble the invadopodium and exhibit high activities of PIK3CA and PIK3CB (36, 37). We found that PH-Tapp1, SHIP2, and SNX9 also concentrated at the invadopodium of MDA-MB-231 cells (Fig. 6 E–G). The knockdown of SHIP2 and SNX9 attenuated their transwell migration abilities (Fig. 6 H–K). Rescues of SHIP2 or SNX9 in each respective knockdown condition restored the invasion capacity. Similarly, the treatment with wortmannin (200 nM, pan PI3K inhibitor) and AS1949490 (10 μM) impeded the transwell migration of MDA-MB-231 cell (SI Appendix, Fig. S5 I and J). Thus, the inhibition of PI3K-SHIP2-SNX9 signaling axis suppresses the invasive cell migration.

Discussion

Phosphoinositide lipids are important signaling messengers to facilitate membrane compartmentalization and trigger a wide variety of cellular events. Here, we dissect the molecular pathway and examine the impact of PI(3,4)P₂ biogenesis on membrane trafficking, integrin receptor endocytosis, and invasive cell migration (Fig. 7). We find that PI(3,4)P₂, unlike other phosphoinositide lipids, is asymmetrically concentrated at the trailing edge of the invadopodium. The PI(3,4)P₂-rich compartment is actively reshaped and forms the invaginated membrane bud and tube in a SNX9- and microtubule-dependent manner, respectively. We further identify regulatory components of membrane tubulation, including AKTIP, Hook1, and microtubule retrograde motor dynein. Functionally, blocking the PI(3,4)P₂-mediated endocytosis pathway impedes integrin endocytosis, prevents the adhesion disassembly, and suppresses the invasive cell migration.

Using the approach of shRNA knockdown and rescue, we find that PIK3CA is critical to generate the intermediate product PI(3,4,5)P₃, which is then converted to PI(3,4)P₂ by the phosphoinositide 5-phosphatase SHIP2. The knockdown and chemical inhibition of PIK3CA result in the decrease of the PI(3,4)P₂ at the invadopodium but do not disrupt the invadopodium formation. Other isoforms of PI3K, such as PIK3CB, may compensate the essential function of PI3K when the expression of PIK3CA is suppressed. Likewise, the knockdown and chemical inhibition of SHIP2 results in the decrease of PI(3,4)P₂.
Fig. 5. PI(3,4)P2-rich membrane invaginations promote the endocytosis of integrin-beta3 receptor. (A) Integrin-beta3-EBFP2 is enriched in the GFP-PH-Tapp1-positive membrane tube behind the invadopodium arc of MEF-Src (arrowheads). (B) More integrin-beta3 puncta, labeled by the conformation-specific LIBS2 antibody, are found in the cytoplasm of invadopodium-forming MEF-Src compared to that of wild-type MEF. Inverted images represent the projection of the confocal z-stack from 1 μm above the adhesion plane to the apex of the cell. (C) The ratio of internalized integrin-beta3 in wild-type MEF and MEF-Src. The statistical information is in SI Appendix, Table S1G. (D) MEF-Src exhibits a higher exocytosis level of integrin-beta3 than wild-type MEF. Inverted images represent the exocytosis level of integrin-beta3 using the antibody-based pulse-chase assay. (E) The intensity level of exocytosed integrin-beta3 in wild-type MEF and MEF-Src. The statistical information is in SI Appendix, Table S1H. (F) The intensity profiles of anti-pY397-FAK and anti-integrin-beta3 (n = 11). (H) The schematic diagram of endosomal pY397-FAK detection. (I) The detection of endosomal pY397-FAK by Western blot. Detached MEF-Src retains a higher level of endosomal pY397-FAK compared to detached wild-type MEF. The treatment with dynasore (20 μM) abolishes the endosomal pY397-FAK level in detached MEF-Src. (J–L) The knockdown of PIK3CA, SHIP2, and SNX9 suppress the level of endosomal pY397-FAK. (M) The turnover of TIRF-converted integrin-beta3-mEOS2 at the invadopodium. When PIK3CA, SHIP2, and SNX9 are knocked down, the photoconverted integrin-beta3-mEOS2 at the invadopodium is more stable than that in the scramble control. (N) The fold of change of photoconverted integrin-beta3-mEOS2 intensity at the invadopodium under the condition as indicated (n = 10 each). The invadopodium arc is labeled by F-actin marker LifeAct-miRFP703 (in A and M) or CF680R-phalloidin (in F). All experiments have been independently repeated three times. The graphs show mean ± SEM. Unpaired two-tailed Student’s t test is applied for the statistical analysis. ** **P < 0.0001 (Scale bars, 10 μm).
Fig. 6. Inhibitions of PI(3,4)P2-mediated membrane invagination suppress the invasive migration of MEF-Src and MDA-MB231 cells. (A) Wild-type MEF and MEF-Src migrate through the Matrigel-coated transwell chamber. MEF-Src invades more than wild-type MEF. (B) The number of invaded wild-type MEF and MEF-Src. The statistical information is in SI Appendix, Table S1I. (C) The knockdown of PIK3CA, SHIP2, and SNX9 suppress the transwell invasion of MEF-Src compared to the scramble control. Reintroducing PIK3CA, SHIP2, and SNX9 restores the invasion of knockdown cells, respectively. (D) The number of invaded MEF-Src cells after knockdown and rescue as indicated. The statistical information is in SI Appendix, Table S1J. (E) GFP-PH-Tapp1 is enriched at the invadopodium of MDA-MB-231 cell (arrowheads). The invadopodium is identified by the F-actin core and the adhesion ring labeled by CF680R-phalloidin and RFP-paxillin, respectively. (F and G) mCherry-SHIP2 and mCherry-SNX9 colocalize with GFP-PH-Tapp1 at the invadopodium of MDA-MB-231 cells (arrowheads). (H and I) Western blot indicates the shRNA knockdown of SHIP2 and SNX9 in MDA-MB-231 cells. (J) The knockdown of SHIP2 and SNX9 suppress the transwell invasion of MDA-MB-231 cells. Reintroducing SHIP2 and SNX9 restores the invasion of knockdown cells. (K) The number of invaded MDA-MB-231 cells after knockdown and rescue as indicated. The statistical information is in SI Appendix, Table S1K. All experiments have been independently repeated three times. The graphs show mean ± SEM. Unpaired two-tailed Student’s t test (in B) and one-way ANOVA with Dunnett’s test (in D and K) are applied. Ns, P > 0.1234; ***P < 0.0002; ****P < 0.0001 (Scale bars, 10 μm).
I. PI3K and SHIP2 promote PI(3,4)P2 production

![Diagram showing PI3K and SHIP2 promoting PI(3,4)P2 production]

II. Membrane bud formation

![Diagram showing SNX9, Hook1, and dynein facilitating membrane tube formation]

III. Membrane tube formation, endocytosis, and recycling

![Diagram showing Arp1/dynein complex, Rab5-positive endosome, and MT growth]

The distributions of SHIP2 and PI(3,4)P2 are remarkably similar and are both enriched at the trailing edge of the invadopodium arc. Although other phosphoinositide 5-phosphatases, such as SYNJ2 and INPPE5, are found at the invadopodium, they can also dephosphorylate PI(4,5)P2, which is the precursor of PI(3,4,5)P3 and indirectly impede the production of PI(3,4)P2 (15, 38, 39). Indeed, we find that the ectopic expression of SYNJ2 or INPPE5 significantly suppresses the production of PI(3,4)P2 at the invadopodium. While SHIP2 plays a critical role in the production and the asymmetrical enrichment of PI(3,4,5)P3 at the invadopodium, SHIP2-mediated PI(3,4)P2 production and requires the PX–BAR domain. PI(3)P and PI(4,5)P2 are not as concentrated as PI(3,4)P2 at the invadopodium. Nevertheless, PI(3)P locally converted from PI(3,4)P2 may also participate in the recruitment and activation of SNX9. Another PI(3,4)P2 effector lamellipodin and N-BAR-domain protein endophilin have been reported to regulate clathrin-independent endocytosis of membrane receptors (44). However, we find that endophilin A1 is mainly located at the leading front, rather than the trailing edge of the invadopodium arc. Intriguingly, SNX9 and DNM2 remain at the membrane bud and the base of the membrane tube. The spatial confinement of SNX9 suggests the implication of nonlipid regulatory factors, such as membrane curvature modulation and protein scaffolding (45, 46).

We find that the formation of micrometer-long uncurved membrane tube is driven by microtubule and the retrograde motor dynein. As the tubulation events often repeatedly occur at the same location, factors that regulate microtubule dynamics, such as Kanks, liprins, and LLSβ, may promote the targeting and stabilization of microtubules to the invadopodium (47). In addition, we find that protein complexes that associate with dynein, including AKTIP and Hook1 (30, 31), are dynamically enriched at the PI(3,4)P2-rich membrane invagination. The elongation speed of the membrane tube can reach 160 nm/s. Previously, the mean speed of recombinant human dynein moving on surface-immobilized microtubules and microtubule sliding over surface-immobilized dynein are 159 ± 60 nm/s and 300 ± 110 nm/s, respectively (48, 49). In general, our observation agrees with the previous measurement. The enrichment of PI(3,4)P2 is known to recruit Tks5 and to activate actin nucleation promoting factor cortactin (22). The slower elongation speed of the membrane tube in the live cell may result from the resistance from membrane tension and de novo F-actin assembly around the invaginated membrane tube that acts as stabilizing coatings and counterbalances the pulling force (50–53). While knockdown of AKTIP does not change the overall microtubule network (54) nor block the endocytosis of integrin receptors, AKTIP is needed for the recruitment of Hook1 to PI(3,4)P2-rich membrane compartment. The absence of Hook1 may locally down-regulate dynein’s motor activity and contribute to the accumulation of stationary PI(3,4)P2-rich vesicles behind the invadopodium arc. Thus, microtubule/dynein-mediated membrane tube formation may accelerate the endocytosis process but does not play an essential role in PI(3,4)P2-mediated endocytosis.

The elevated expression of SNX9 is often found in the metastatic mammary cancer (55). While the knockdown of SNX9 promotes the matrix degradation by suppressing the endocytosis of matrix metalloproteinase MT1-MMP, SNX9-depleted cells still exhibit the migration defect (56). Thus, apart from controlling the matrix degradation and MT1-MMP endocytosis, SNX9 must have other functional roles in regulating the cell motility. Indeed, SNX9 positively stimulates the angiogenesis by promoting the integrin trafficking in human umbilical vein endothelial cells (57). Here, we also find that the knockdown of SNX9 blocks integrin-beta3 endocytosis, suppresses the adhesion turnover, and impedes the invasion through a Matrigel-coated transwell chamber. Membrane receptors involved in the invadopodium, such as CD44, discoidin domain receptor, and other integrins, are likely to be regulated in the SNX9- and PI(3,4)P2-dependent endocytosis pathway.

Invadopodia, like macrophage podosomes and focal adhesions, are integrin-mediated adhesions at the cell–matrix interface. The composition and the distribution of phosphoinositide lipids can play diverse roles in regulating the signaling event at the adhesion site. At the focal adhesion, the local production of PI(4,5)P2 by PIPKIγ90 facilitates the recruitment of talin and kindlin, which contribute to the integrin activation (58). Likewise, the biogenesis of PI(3,4,5)P3 acts as the key factor to promote WASP...
activation at the macropodeme was observed through imaging. At the invadopodial stage, enrichment of PI(3,4)P₂ and PI(3,4,5)P₃ is known to support F-actin stabilization by interacting with Tks5 and Myo1e, respectively (19, 22). Here, we report a functional role of PI(3,4)P₂ in promoting the SNX9 and microtubule-mediated membrane invagination and integrin trafficking. The turnover and recycling of integrin receptors are crucial for the cell migration. Factors that impede the PI(3,4)P₂ signaling and the membrane invagination may provide insights in inhibiting the cancer metastasis.

Materials and Methods

Cell Culture. RPTPα−/− MEF cell line was a gift from Dr. Sap Jan, New York University School of Medicine. MDA-MB-231 cell line was obtained from the American Type Culture Collection. MEF, MEF-Src (MEF expressing the constitutively activated Src-Y530F mutant), and MDA-MB-231 cell lines were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS (vol/vol), 100 unit/mL penicillin, and 100 μg/mL streptomycin (Thermo Fisher Scientific) in a 37 °C incubator with 5% CO₂. Unless otherwise stated, MEF-Src cells and the substrate coated with fibronectin (Thermo Fisher Scientific, 33016015) were used in the experiment.

Chemical Inhibitor. AS1949940 (3718) and dynasore (2897) were purchased from Tocris Bioscience. A66 (S2636), nocodazole (S2775), and wortmannin (S2798) were purchased from Selleckchem. The chemicals were dissolved in DMSO as a stock, except in the case of stock concentrations 1,000 times higher than the working concentration. Before the application, the chemicals were then diluted to the working concentration with DMEM.


Fluorescence Microscopy. Fluorescent images were taken by an inverted spinning-disc confocal microscope or an inverted TIRF microscope. The spinning-disc confocal microscope (Nikon Ti-P view城际, Perkin-Elmer Ultraview VoX and Yokogawa CSU-X1) was controlled by Volocity software and was equipped with an electron-multiplying charge-coupled device (EMCCD) camera (Hamamatsu C9100-2388), a 100x oil immersion lens (numerical aperture [NA] = 1.45), acousto-optic tunable filter (AOTF)-controlled solid-state lasers (40 to 50 mW), and a piezo Z stage. The TIRF microscope (Zeiss Axio Observer with Las2 TIRF module) was operated by MetaMorph software (Molecular Devices) and was equipped with an EMCCD camera (Photometrics Evolve 512), a 100x oil immersion lens (NA = 0.70), and AOTF-controlled solid-state lasers (50 to 100 mW). Emission filter sets were 445W/60/515W/70 (dual band-pass; EBF4 and mCherry), 527W/545 (EGFP), 485W/60/705W/90 (dual band-pass; cyan fluorescent protein and far-red probe). An environmental chamber (37 °C and 5% CO₂) was attached to the microscope for long-term time-lapse imaging. Phenol red-free DMEM with 100 μM L-glutamine, 100 μM di-nitrophenylarginine, and 20 mM HEPES was used as the imaging buffer for live-cell microscopy. Typically, the low laser power (less than 10%) and short exposure time (100 ms) were used to avoid nonspecific phototoxicity and pixel saturation. To avoid the overexpression artifact, cells with low expression level of fluorescent protein were targeted. To avoid signal from the nucleus. The FOI of the protein of interest at the invadopodium was defined as Icore (Icytosol Ibackground)−(Icytosol Ibottommost background). The FOI at the plasma membrane was also measured in ImageJ software. The ratio of internalized integrin-β3 was defined as (Icore − Icytosol)/(Icytosol − Ibackground)−(Icytosol − Ibottommost background Ibottommost background) and plotted using GraphPad Prism software.

Exocytosis Assay. MEF and MEF-Src cells were transfected with the human integrin-β3 plasmid and plated on the fibronectin-coated glass. After 48 h, cells were cooled to 4 °C in serum-free DMEM containing 0.1% BSA and incubated with human integrin-β3 antibody (LIB2) for 1 h. The unbound antibody was washed away by serum-free DMEM at 4 °C. Cells were then maintained in the condition of serum-free DMEM containing 0.1% BSA for 2 h at 37 °C, in which the endocytosis of antibody-bound integrin-β3 took place. Subsequently, the acid wash (0.5% glacial acetic acid and 0.5 M NaCl in pH = 3) was applied to remove the residual antibody left on the plasma membrane. The exocytosis was stimulated by replacing the culture medium with DMEM containing 10% FBS. After 5 min of serum stimulation, the cells were fixed, stained with secondary antibody and Cy3 phalloidin, and then imaged by the confocal microscopy. Identical experiments without the initial incubation of LIB2 were performed and acted as the control. The exocytosis level of integrin-β3 receptor was determined by subtracting the intensity of the secondary antibody on the bottom layer of plasma membrane (summed between 0 and 400 nm, 200 nm per stack) of the control cells from that of the LIB2-treated cells.

Photoswitchable Integrin-β3-mEOS2 and Adhesion Turnover. A single photoactivation of 405 nm light by TIRF microscopy was applied to MEF-Src at the beginning in order to specifically convert the integrin-β3-mEOS2 receptor at the cell–matrix interface. The MEF-Src cells with photoswitched integrin-β3-mEOS2 (Set #1) were then imaged by the excitation of 561 nm laser and the emission filter of mCherry (615W70) every 3 min for 78 min (total 27 exposure counts). To measure the effect of photobleaching, another set of MEF-Src cells with the similar expression level of integrin-β3-mEOS2 (Set #2) were also imaged 27 times with the same microscopy settings and a shorter time interval of 5 s. In both Set #1 and #2, the mean intensity of photoswitched integrin-β3-mEOS2 within the ROI of the invadopodium (Iinvadopodium) and the mean intensity of noncellular background (Ibackground) was measured in ImageJ software, and the dynamic changes of intensity profiles were consistent across multiple examples at similar temporal points.

Quantification of Lateral Distribution Analysis. The invadopodium arc was identified by the densely polymerized F-actin and imaged at the bottommost adhesion plane that was indicated by the plasma membrane marker CAAX. Unless otherwise stated, outside-in intensity line scans across the invadopodium were applied to measure the distribution of the protein of interest in ImageJ software. To compare the relative distribution among various lipid probes, acquired data sets were realigned so that the maximum fluorescence intensity in the F-actin channel was fixed at the position of the 101st pixel along the line scan. After normalization, the lateral distribution of the protein of interest relative to that of F-actin was then compared and plotted using GraphPad Prism software. In the quantification of membrane tube extension, the analysis of single representative cell was shown, and the dynamic changes of intensity profiles were consistent across multiple examples at similar temporal points.
(background) were quantified using Imaris software. The net intensity \( I_{\text{net-px}} \) was defined by subtracting \( I_{\text{background}} \) from \( I_{\text{px}} \). In Set #2, the bleach correction function was generated by fitting the intensity changes of \( I_{\text{px-Set2}} \) with a two-phase decay model in GraphPad Prism software. In Set #1, the bleach-corrected intensity was then determined by the division of \( I_{\text{px-Set1}} \) by the bleach correction function and was plotted in GraphPad Prism software. Pseudocolor ratio images were prepared in ImageJ software using the same calculation above, and the identical dynamic range was applied.

**Statistical Tests.** All datasets contained at least three independent biological repeats. Statistical graphs with the mean and SEM were plotted using GraphPad Prism software. Statistical tests, including unpaired two-tailed Student’s t-test and one-way ANOVA with Dunnett’s test, were performed using GraphPad Prism software. Detailed statistical information and P values can be found in SI Appendix, Table S1. Not significant (ns), \( P > 0.1234 \), \( *P < 0.001 \), ***\( P < 0.0002 \), and ****\( P < 0.0001 \).

**Data Availability.** All study data are included in the article and/or supporting information.

**ACKNOWLEDGMENTS.** This work was supported by Research Grant Council of Hong Kong, ECS 27118615 and GRF 17118620 (C.-h.Y.). We also thank Imaging and Flow Cytometry Core and Bioreagent Core in Centre for PanBioMics Sciences in Faculty of Medicine, University of Hong Kong.