α5β1 integrin trafficking and Rac activation are regulated by APPL1 in a Rab5-dependent manner to inhibit cell migration

Nicole L. Diggins¹,*, Hakmook Kang², Alissa Weaver³,⁴,⁵ and Donna J. Webb¹,⁵,⁶,‡

ABSTRACT

Cell migration is a tightly coordinated process that requires the spatiotemporal regulation of many molecular components. Because adaptor proteins can serve as integrators of cellular events, they are being increasingly studied as regulators of cell migration. The adaptor protein containing a pleckstrin-homology (PH) domain, phosphotyrosine binding (PTB) domain, and leucine zipper motif 1 (APPL1) is a 709 amino acid endosomal protein that plays a role in cell proliferation and survival as well as endosomal trafficking and signaling. However, its function in regulating cell migration is poorly understood. Here, we show that APPL1 hinders cell migration by modulating both trafficking and signaling events controlled by Rab5 in cancer cells. APPL1 decreases internalization and increases recycling of α5β1 integrin, leading to higher levels of α5β1 integrin at the cell surface that hinder adhesion dynamics. Furthermore, APPL1 decreases the activity of the GTPase Rac and its effector PAK, which in turn regulate cell migration. Thus, we demonstrate a novel role for the interaction between APPL1 and Rab5 in governing crosstalk between signaling and trafficking pathways on endosomes to affect cancer cell migration.

This article has an associated First Person interview with the first author of the paper.

KEY WORDS: Integrin trafficking, Cell motility, Extracellular matrix, 3D migration, Endosomes, p21-activated kinase

INTRODUCTION

The establishment of cell polarity requires vesicular trafficking of proteins to distinct cellular locations, which is crucial for cell migration (Jacquemet et al., 2013b; Maritzen et al., 2015). Cell migration is marked by four key steps: extension of an actin-rich protrusion; establishment of cell–matrix adhesions; translocation of the cell body; and retraction of the cell rear (Borisy and Svitkina, 2000; Ridley, 2011; Ridley et al., 2003). Each step requires spatiotemporal regulation of protein transport. For example, adhesions, which link the extracellular matrix (ECM) to the actin cytoskeleton, must continuously assemble and disassemble – a process termed adhesion turnover – for cells to migrate efficiently (Broussard et al., 2008; Lock et al., 2008; Vicente-Manzaneques and Horwitz, 2011; Wehrle-Haller, 2012). Trafficking of key adhesion proteins, particularly integrins, through the early endocytic pathway is emerging as an important mechanism for regulating adhesion dynamics (Paul et al., 2015).

Integrins are constitutively endocytosed and recycled back to the cell surface of migrating cells (Bretscher, 1989, 1992; Caswell and Norman, 2006; Lawson and Maxfield, 1995). Integrin trafficking is spatiotemporally regulated to target integrins to adhesions (Caswell and Norman, 2006) and regulate adhesion turnover (Chen et al., 2014; Mendoza et al., 2013; Pellinen et al., 2006). The internalization and recycling of integrins through the endocytic pathway have been intensely studied (Caswell et al., 2007; Dozynkiewicz et al., 2012; Lobert et al., 2010; Tiwari et al., 2011; White et al., 2007), yet there are still questions remaining as to which endocytic proteins are involved in this process. Rab5 is considered to be a master regulator of early endocytic events, controlling cargo recruitment, fusion and motility of early endosomes (van der Bliek, 2005). In addition, Rab5 promotes cell migration by regulating vesicular trafficking and localization of proteins involved in migration (Mendoza et al., 2014; Stupack and Torres, 2011). Intriguingly, Rab5 associates with focal adhesion proteins and α- and β-integrins, and promotes the disassembly of adhesions, potentially by promoting the internalization of integrins (Mendoza et al., 2013; Pellinen et al., 2006). Moreover, Rab5-mediated trafficking regulates signaling events, as Rab5-dependent endocytosis has been shown to activate the small GTPase Rac on endosomes through the guanine nucleotide exchange factor (GEF) Tiam1 (Diaz et al., 2014; Palamidessi et al., 2008; Torres et al., 2010). Active Rac associates with effectors, including p21-activated kinase (PAK), to promote actin polymerization (Szczepanska, 2009). Re-localization of active Rac to the leading edge by Rab5 induces the forward protrusion of the membrane necessary for migration (Palamidessi et al., 2008). Through its regulation of trafficking events, Rab5 promotes tumor cell invasion and metastasis (Christoforides et al., 2012; Kawauchi, 2012; Mendoza et al., 2014; Stupack and Torres, 2011), further suggesting that endocytic trafficking through Rab5 is important for regulating cell migration.

While Rab5 is known to induce cell migration through endocytic trafficking, the effectors in this process are not well understood. The adaptor protein containing a pleckstrin-homology (PH) domain, phosphotyrosine binding (PTB) domain, and leucine zipper motif 1 (APPL1) is a 709 amino acid protein that localizes to early endosomes (Li et al., 2007) and interacts with Rab5 (Zhu et al., 2007). APPL1 is composed of several domains. The N-terminal Bin–Amphiphysin–Rvs (BAR) domain allows APPL1 to form homodimers or heterodimers with APPL2 (Chial et al., 2010; Zhu et al., 2007). The central pleckstrin homology (PH) domain binds phosphoinositol lipids and may be partially responsible for...
association of APPL1 with endosomal membranes (Li et al., 2007; Zhu et al., 2007). The BAR and PH domains interact to form a functional BAR-PH domain that mediates the binding of Rab5 (Zhu et al., 2007). The C-terminal phosphotyrosine-binding (PTB) domain binds signaling proteins including Akt (Mitsuuchi et al., 1999; Schenck et al., 2008) and a variety of receptors (Deepa and Dong, 2009; Lin et al., 2006; Liu et al., 2002; Varsano et al., 2012). The SEA motif on the C-terminus of APPL1 binds to GAP-interacting protein C terminus, member 1 (GIPC1) (Lin et al., 2006), a protein involved in loading cargoes onto vesicles (Aschenbrenner et al., 2003), suggesting a role for APPL1 in cellular trafficking.

APPL1 mediates cellular functions including cell survival, cell growth and proliferation (Miaczynska et al., 2004; Schenck et al., 2008; Tan et al., 2010). Interestingly, APPL1 has also been implicated in cell migration (Diggins and Webb, 2017). Our group has previously shown that APPL1 expression hinders cell migration by reducing Akt activation, thereby impairing adhesion turnover (Broussard et al., 2012). In addition, studies employing a genetic approach show that both APPL1 and APPL2 are necessary for hepatocyte growth factor (HGF)-induced migration of murine embryonic fibroblasts (MEFs) (Tan et al., 2016, 2010). A recent study demonstrated that a subset of Arf6 compartments that colocalizes with APPL1 mediates recycling of β1 integrins, suggesting that APPL1 may be involved in integrin trafficking (Chen et al., 2014). Whether APPL1 is directly involved in the regulation of integrin trafficking or merely present on compartments containing integrins is currently unknown.

In this study, we demonstrate that APPL1 decreases the rate of α5β1 integrin internalization and increases the rate of recycling to promote higher cell surface levels of integrin. In addition, APPL1 decreases activation of Rac and its effector PAK in a Rab5-dependent manner. Therefore, we have identified a new mechanism by which APPL1 modulates migration via α5β1 integrin trafficking and Rac signaling, mediated by Rab5.

RESULTS
The endosomal protein APPL1 is a negative regulator of cell migration
To begin investigating the role of APPL1 in cell migration, HT1080 fibrosarcoma cells were transfected with constructs expressing either APPL1-GFP or GFP, plated on fibronectin (FN)-coated cell culture dishes, and migration assays were performed. APPL1-GFP was overexpressed approximately 2.8-fold over endogenous levels of APPL1 (Fig. 1A). We observed that APPL1-GFP-expressing cells migrated significantly shorter distances, compared with the control (Fig. 1B; Movies 1 and 2), suggesting that APPL1 inhibits cell migration. Quantification of the migration speed showed a ~30% decrease in APPL1-GFP-expressing cells compared with control cells (Fig. 1C). Notably, analysis of mean squared displacement (MSD) showed that APPL1-GFP-expressing cells explored significantly less area than GFP-expressing cells did (Fig. 1D). This difference in MSD is probably due to the decrease in migration speed, as cells expressing APPL1-GFP showed no difference in persistence, quantified as the distance traveled divided by the total path travelled (Fig. 1E). Additionally, direction autocorrelation was calculated as an unbiased measure of directionality by compensating for differences in speed (Gorelik and Gautreau, 2014), and showed no difference between APPL1-GFP- and GFP-expressing cells (Fig. 1F). To determine whether this effect is specific to HT1080 cells or represents a more general mechanism, we also performed migration assays with MDA-MB-231 cells. As with HT1080 cells, expression of APPL1-GFP in MDA-MB-231 cells led to significantly shorter migration distances (Fig. S1A) and a 62.7% decrease in speed compared with levels in the control (Fig. S1B). Moreover, MDA-MB-231 cells expressing APPL1-GFP had decreased MSD (Fig. S1C), but no change in persistence (Fig. S1D) or directionality (Fig. S1E), compared with that in the control. To examine the effects of APPL1 on migration in a more in vivo-like environment, we performed migration assays in 3D collagen I (Coll) gels. HT1080 cells expressing APPL1-GFP or GFP were embedded in Coll gels supplemented with FN and allowed to adhere overnight. Similar to results observed in 2D migration, APPL1-GFP-expressing cells migrated ~25% slower than cells expressing GFP in 3D matrices (Fig. S1F; Movies 3 and 4), and exhibited decreased MSD (Fig. S1G). However, there was no change in persistence (Fig. S1H) or directionality (Fig. S1I) between GFP- and APPL1-GFP-expressing cells in 3D matrices. Together, these data indicate a role for APPL1 in modulating cell migration.

To explore further the role of APPL1 in regulating cell migration, we used the CRISPR/Cas9 system to generate frame-shift mutations in APPL1. HT1080 cells were transfected with plasmids expressing Cas9 and one of three different guide RNAs (gRNA) or a non-targeting (NT) gRNA as a control. Cells containing the plasmid were selected by puromycin resistance, which resulted in a mixed population of cells with various insertions/deletions (INDELs) in the APPL1 gene. As anticipated, cells expressing each gRNA (APPL1 gRNA#1-3) showed an ~85–90% reduction in APPL1 expression, compared with NT gRNA-expressing cells, indicating that the CRISPR/Cas9 system was effective for greatly diminishing APPL1 expression (Fig. 1G,H). Migration assays were performed using APPL1 gRNA-expressing cells or control cells. APPL1 gRNA-expressing cells had longer migration paths compared with control cells (Fig. 1I). APPL1 gRNA#1 led to a ~1.3-fold increase in migration speed, while APPL1 gRNA#2 and APPL1 gRNA#3 led to a ~1.4-fold increase in migration speed, compared with migration speed of control cells (Fig. 1J). Expression of all three guide RNAs resulted in a increased MSD compared with that in the non-targeting control (Fig. 1K), but no difference in persistence (Fig. 1L) or directionality (Fig. 1M). Since all three gRNAs had similar effects on APPL1 expression and cell migration (Fig. 1J), APPL1 gRNA#3 cells were utilized for all subsequent experiments. In order to test whether APPL2 also plays a role in cell migration, APPL1 gRNA#3 or NT gRNA cells were transfected with a siRNA pool targeted against APPL2, resulting in a 50% decrease in APPL2 expression (Fig. S1J,K). No difference in migration speed was observed in cells depleted of APPL2 alone or in combination with depletion of APPL1 (Fig. S1L). Overall, these results suggest that APPL1 is an important regulator of cell migration.

Regulation of cell migration by APPL1 depends on α5 integrin
Our previous work has shown that some regulators of cell migration act in an ECM-specific manner (Bristow et al., 2009; Jean et al., 2014). Since APPL1 regulates 3D migration (Fig. S1F), a situation in which cells are in the presence of both Coll and FN, we wanted to test whether APPL1-mediated migration is ECM dependent. Migration assays were performed with HT1080 cells expressing APPL1-GFP or GFP and plated on either FN or Coll. While APPL1-GFP-expressing cells showed a decreased migration speed on FN, APPL1 had no effect on migration speed on Coll (Fig. 2A). Likewise, APPL1 gRNA#3 cells increased their speed of migration when plated on FN, but not Coll (Fig. 2B), suggesting that APPL1 may regulate migration in a manner dependent on α5β1, a major FN-binding integrin. Three-dimensional migration assays were performed in the presence of the
Fig. 1. APPL1 decreases cell migration. (A) Lysates derived from cells expressing GFP or APPL1-GFP were subjected to immunoblot analysis for levels of APPL1 and β-actin (loading control). A representative image is shown. M, molecular weight marker. (B) Migration was quantified for individual cells expressing either GFP or APPL1-GFP. Rose plots show individual tracks of representative cells from each condition. (C) Box plot showing migration speeds for GFP- and APPL1-GFP-expressing cells; 59–66 cells were analyzed from each condition from at least three experiments. (D–F) MSD (D) persistence index (E) and directional autocorrelation analysis (F) of HT1080 cells expressing GFP or APPL1-GFP. Error bars represent s.e.m. from 44–46 cells total from each condition from at least three separate experiments. [***P < 0.0001, comparing slopes of the lines using a likelihood ratio test (D), n.s., not significant determined by Student’s t-test (P = 0.48; E), or determined by mixed effects model to compare the curves (P = 0.353), with controlling false discovery rate (FDR) at 0.1 (F)]. (G) Lysates derived from cells expressing one of three different CRISPR plasmids targeting APPL1 (APPL1 gRNA#1-3) or non-targeting guide RNA (NT gRNA) were subjected to immunoblot analysis for endogenous levels of APPL1 and β-actin (loading control). A representative image is shown. (H) Quantification of the endogenous levels of APPL1 in the blots described in G, normalized to β-actin. Error bars represent the s.e.m. from three separate experiments (***P < 0.0001, determined by one-way ANOVA). (I) Rose plots from migration assays show the individual track paths for APPL1 gRNA cell lines or control cells. (J) Box plot showing migration speed for the indicated cell lines; >60 cells total were analyzed for each condition from at least three experiments (***P < 0.0001, determined by one-way ANOVA). All box plots range from the 25th to 75th percentile, the line indicates the median, and whiskers show the minimum and maximum. (K–M) MSD (K), persistence index (L), and directional autocorrelation analysis (M) of HT1080 cells expressing APPL1 gRNA#1-3 or NT gRNA. Error bars represent s.e.m. from >60 cells total from each condition from at least three separate experiments (***P < 0.0001, comparing slopes of the lines using a likelihood ratio test (K), n.s., not significant (P = 0.48) determined by one-way ANOVA (L), P = 0.115, 0.404, and 0.044, NT gRNA compared with gRNA #1, gRNA #2, and gRNA #3, respectively, determined by mixed-effects model to compare the curves, with controlling FDR at 0.1 (M)].
synthetic peptide RGD (10 μM) to block integrin–ligand interactions or an equal concentration of RGE peptide as a control. Treatment with RGD did not disrupt attachment of GFP- or APPL1-GFP-expressing cells in the ColI gels (Fig. S1M). Consistent with our previous results, APPL1-GFP-expressing cells migrated more slowly than control cells in the presence of RGE (control) peptide, whereas the presence
Fig. 2. APPL1 impairs migration by increasing cell surface levels of α5 integrin. (A,B) Box plot showing migration speed for GFP- or APPL1-GFP-expressing cells (A) or cells expressing APPL1 gRNA#3 or NT grNA (B) plated on either FN or Coll substrate. At least 25 cells (A) or at least 55 cells (B) total were analyzed from each condition from at least three separate experiments (**P<0.005, ***P<0.0001, n.s., not significant, P=0.41 (A) and P=0.57 (B) determined by Student’s t-test). (C) Box plot shows migration speed for GFP- or APPL1-expressing cells in 3D migration assays treated with α5 integrin function-blocking antibody (P1D6) or control antibody (IgG); 18–31 cells total were analyzed from each condition from three separate experiments (**P<0.005, ***P<0.0001, n.s., not significant, P=0.79 and P>0.99, comparing GFP control to GFP and APPL1-GFP treated with P1D6, respectively, determined by one-way ANOVA followed by Tukey’s post hoc test). (D,G) HT1080 cells expressing GFP or APPL1-GFP (D) or NT grNA or APPL1 gRNA#3 (G) were surface labeled with NHS-SS-Biotin and pulled down with streptavidin. Surface (pulldown) and total (whole-cell lysate, WCL) samples were immunoblotted for α5, β1 or β3 integrin and β-actin. A representative image is shown. M, molecular weight marker. (E,F,H,I) Quantification of total (E,H) or surface (F,I) α5, β1 or β3 integrin from D and G, respectively, shown as a percentage of control. Error bars represent s.e.m. from at least three separate experiments (*P<0.05, n.s., not significant, P=0.35, 0.36 and 0.32 (E), P=0.56 (F), P=0.24, 0.83 and 0.46 (H), and P=0.26 (I)). (J,L,N,P) HT1080 cells expressing GFP or APPL1-GFP (J,L) or APPL1 gRNA#3 or non-targeting (NT) grNA (N,P) were immunostained for total α5 integrin (clone 6F4) (J) or active α5 integrin (clone SNKAS1) (L,P). TIRF microscopy was performed to visualize surface level expression. Representative images are pseudocolored for intensity, where warm colors represent higher intensity and cool colors represent lower intensity. Scale bars: 20 μm. (K,M,O,Q) Quantification of α5 integrin levels from J,L and N, P, respectively, shown as a percentage of control. Error bars represent the s.e.m. from 55–66 cells total from three separate experiments (*P<0.05, **P<0.005, ***P<0.0005, determined by Student’s t-test).

The presence of RGD abrogated the effect of APPL1 on cell migration (Fig. S1N). The RGD peptide blocks the function of multiple integrins, not just α5β1. To verify specificity, we evaluated migration speeds in 3D migration assays while treating with an anti-α5 integrin function-blocking antibody (clone P1D6) or control IgG antibody. Treatment with P1D6 had no effect on attachment of GFP- or APPL1-GFP-expressing cells in the 3D Coll gel (Fig. S1O). As expected, APPL1-GFP-expressing cells migrated significantly more slowly in the presence of the control antibody, but no difference in migration speed was observed when APPL1-GFP-expressing cells were treated with P1D6 antibody (Fig. 2C). These results suggest that the effect of APPL1 on cell migration is dependent on α5β1 integrin.

We hypothesized that APPL1 could affect surface level expression of α5β1 integrin to mediate cell migration. To address this possibility, APPL1-GFP or GFP-expressing cells were surface labeled with biotin, and labeled proteins were pulled down using streptavidin agarose beads and subjected to western blot analysis. Whole-cell lysates from GFP- and APPL1-GFP-expressing cells showed no difference in total expression levels of α5, β1 or β3 integrin (Fig. 2D,E). However, APPL1-GFP expression led to increased surface levels of both α5 and β1 integrin, but not β3 integrin (Fig. 2D,F). Conversely, APPL1 gRNA#3 cells had levels of total α5, β1 and β3 integrin similar to those in NT grNA cells (Fig. 2G,H), yet had decreased surface levels of α5 and β1 (but not β3) integrin (Fig. 2J). To confirm these results, HT1080 cells expressing APPL1-GFP or GFP were immunostained for both total and active α5 integrin (clones 6F4 and SNKAS1, respectively). Cell surface integrins were visualized by total internal reflection (TIRF) microscopy. Cells expressing APPL1-GFP exhibited higher levels of both total (Fig. 2I,K) and active (Fig. 2L,M) α5 integrin, compared with control cells. Conversely, decreasing endogenous APPL1 expression through expression of APPL1 gRNA#3 reduced surface levels of both total (Fig. 2N,O) and active (Fig. 2P,Q) α5 integrin, suggesting that APPL1 promotes higher cell surface levels of α5β1 integrin to inhibit cell migration.

We further investigated the relationship between APPL1 and α5 integrin by assessing α5 integrin dynamics. HT1080 cells were co-transfected with a photoactivatable GFP-tagged α5 integrin (α5-PA-GFP) and either APPL1-mCherry or mCherry (control). Live cell imaging experiments were performed in which a region of interest (ROI) was selected at the edge of an mCherry-positive cell, and then the fluorescence of α5-PA-GFP was activated by a 500 ms pulse of 405 nm light. Following photoactivation, fluorescence in control cells quickly dissipated, demonstrating rapid movement of α5 integrin away from the edge of the cell, either through lateral movement or internalization. However, APPL1-mCherry-expressing cells retained significantly higher levels of fluorescence over time, indicating that α5 integrin has slower dynamics as a result of increased APPL1 expression (Fig. 3A,B). APPL1 has been shown to regulate adhesion dynamics (Broussard et al., 2012), so we next assessed whether α5 integrin dynamics were altered in adhesions. Similar experiments were performed with NT grNA or APPL1 grNA#3 cells transfected with α5-PA-GFP and mCherry-paxillin, an adhesion marker. Regions of interest were chosen at sites of adhesions, where APPL1 grNA#3 cells showed faster α5 integrin dynamics, compared with levels in NT grNA cells (Fig. 3C,D). It is possible that APPL1 could alter the dynamics of receptors other than just α5 integrin; therefore, transferrin receptor (TfnR) was tested. Unlike our results with α5 integrin, cells expressing APPL1-GFP showed no difference in TfnR dynamics compared with control cells (Fig. S2A). Taken together, these results suggest that APPL1 inhibits cell migration by diminishing α5 integrin movement away from adhesions.

APPL1 regulates α5β1 integrin trafficking

Because APPL1 hindered the dynamics of α5 integrin at the cell edge and APPL1 is involved in vesicular trafficking, we hypothesized that APPL1 affects α5β1 integrin trafficking. We performed biotinylation internalization assays, where HT1080 cells expressing APPL1-GFP or GFP were surface labeled with NHS-SS-biotin and internalization was allowed to occur for 0–60 min. Surface-associated biotin was removed by TCEP, a reducing agent, biotinylated (internal) proteins were pulled down using streptavidin, and western blots were performed with NT grNA or APPL1 gRNA#3 cells showing faster α5 integrin dynamics, compared with levels in NT grNA cells (Fig. S3A,B). It is possible that APPL1 could alter the dynamics of receptors other than just α5 integrin; therefore, transferrin receptor (TfnR) was tested. Unlike our results with α5 integrin, cells expressing APPL1-GFP showed no difference in TfnR dynamics compared with control cells (Fig. S2A). Taken together, these results suggest that APPL1 inhibits cell migration by diminishing α5 integrin movement away from adhesions.
**Fig. 3.** APPL1 alters α5 integrin dynamics. (A) HT1080 cells expressing α5-PA-GFP and either mCherry or APPL1-mCherry were subjected to live-cell photoactivation experiments. Images from selected time points are shown and are pseudocolored for intensity. Circle demarcates ROI used for photoactivation and subsequent average fluorescence intensity measurements. Dotted lines indicate the boundaries of the cell. Scale bar: 15 μm. (B) Quantification of experiments from A as average fluorescence intensity, normalized to the amount of signal within the ROI at time=0. (C) HT1080 cells expressing APPL1 gRNA#3 or NT gRNA were transfected with α5-PA-GFP and mCherry-paxillin and subjected to live-cell photoactivation experiments. White boxes in top images indicate regions shown at higher magnification in bottom panels. Bottom left, zoomed images from top panel showing mCherry-paxillin. Arrows indicate adhesions chosen as ROI for photoactivation. Bottom right, images showing α5-PA-GFP fluorescence from selected time points, pseudocolored for intensity. White circles represent ROI used for quantification. Scale bars: 15 μm (top) and 5 μm (bottom). (D) Quantification of photoactivation experiments from C. Error bars represent the s.e.m. of 14–15 cells (B) or 27–28 cells (D) from each condition from three separate experiments. A nonparametric regression analysis was used to fit the curves (using natural cubic splines with three knots) and then the difference in the curves compared between two groups using the likelihood ratio test (**P<0.001).
Fig. 4. APPL1 regulates α5β1 integrin internalization and recycling. (A) HT1080 cells expressing GFP or APPL1-GFP were surface labeled with NHS-SS-Biotin for 30 min at 4°C and biotinylation internalization assays were performed. Surface and internalized α5 and β1 integrin were assessed by western blot analysis. A representative image is shown. M, molecular weight marker. (B,C) Quantification of β1 integrin (B) or α5 integrin (C) internalization from A, shown as a percentage of surface integrin. Error bars represent s.e.m. from at least three separate experiments (*P<0.05, determined by Student’s t-test). (D) HT1080 cells expressing APPL1 gRNA#3 or non-targeting (NT) gRNA were transfected with GFP, APPL1-GFP, APPL1-N308D-GFP or APPL1-ΔPTB-GFP and then were surface labeled with NHS-SS-Biotin and internalization assays were performed. A representative image is shown. (E,F) Quantification of β1 integrin (E) or α5 integrin (F) internalization from D shown as a percentage of surface integrin. Error bars represent s.e.m. from three separate experiments (*P<0.05, determined by one-way ANOVA, comparing NTgRNA+GFP to gRNA3+GFP). (G) HT1080 cells expressing GFP or APPL1-GFP were surface labeled with NHS-SS-Biotin for 30 min at 4°C and biotinylation recycling assays were performed. Western blot analysis was used to assess the remaining internal pool of α5β1 integrin. A representative image is shown. (H,I) Quantification of β1 integrin (H) or α5 integrin (I) recycling from G as percentage recycled (0% recycled at time=0). Error bars represent s.e.m. from three separate experiments (*P<0.05, determined by Student’s t-test).
APPL1 gRNA#3 cells exhibited increased internalization of β1 (Fig. 4D,E) and α5 integrin (Fig. 4D,F), compared with control cells. However, rescue with APPL1-GFP, but not APPL1-N308D-GFP or APPL1-ΔPTB-GFP, in APPL1 gRNA#3 cells resulted in internal integrin levels similar to those in NT gRNA cells (Fig. 4E,F), suggesting that APPL1 expression can modulate integrin internalization, dependent on its interaction with Rab5 and its PTB domain.

To ensure that APPL1 was not grossly affecting trafficking, internalization assays were performed to assess TfnR trafficking. Transferrin conjugated to a pH-sensitive dye pHrodo Red, was added to cell culture media of GFP- or APPL1-GFP-expressing HT1080 cells. Since pHrodo Red dye is non-fluorescent at neutral pH but fluoresces brightly in acidic environments like endosomes, fluorescence is proportional to the amount of internalized TfnR–Tfn complex. No difference in internal TfnR levels between GFP- or APPL1-GFP-expressing cells was observed, suggesting that APPL1 does not alter TfnR trafficking (Fig. S2B,C). Overall, these results suggest that APPL1 decreases internal levels of β1 integrin, but not TfnR.

Lower internal integrin levels could result from a decreased rate of internalization or from an increased rate of recycling. Therefore, we performed antibody internalization assays in the presence of primaquine, a recycling inhibitor. In the presence of primaquine, APPL1-GFP-expressing cells still exhibited lower internal levels compared with levels in the control, suggesting that APPL1 decreases β1 integrin internalization (Fig. S3G). Next, we directly assessed the ability of APPL1 to modulate integrin recycling. Antibody recycling assays were performed in which cells were treated with primaquine during the internalization step to prevent recycling, surface antibody was removed by acid wash, and then internal integrin was chased back to the cell surface by returning cells to 37°C for 0–20 min in the absence of primaquine, followed by a second acid wash to remove antibody recycled to the cell surface. Intriguingly, APPL1-GFP-expressing cells recycled β1 integrin significantly faster compared with GFP-expressing cells (Fig. S3H,I). We confirmed these results biochemically, and observed similar results to the immunofluorescence method used to probe integrin recycling (Fig. 4G–I). Collectively, these data demonstrate that APPL1 both decreases integrin internalization and promotes integrin recycling, leading to lower internal levels of β1 integrin.

**APPL1 inhibits cell migration in a Rab5-dependent manner**

APPL1 was previously shown to interact with the GTPase Rab5 on early endosomes (Zhu et al., 2007) and our results indicate that APPL1 affects integrin trafficking. We hypothesized that endosomal localization of APPL1 is important for its effect on cell migration. Migration assays were performed using an APPL1 variant [APPL1-R146A/K152A/K154A (APPL1-AAA-GFP)] that has three point mutations in the BAR domain that abolish endosomal localization (Broussard et al., 2012; Schenck et al., 2008). Whereas APPL1-GFP localized to punctate endosomal structures, APPL1-AAA-GFP showed diffuse localization (Fig. S4A), consistent with previous findings (Broussard et al., 2012). While APPL1-GFP expression hindered cell migration in HT1080 cells, APPL1-AAA-GFP-expressing cells migrated similar distances (Fig. 5A) and speeds (Fig. 5B) compared with the control cells. Analogous results were obtained in MDA-MBA-231 cells expressing APPL1-AAA-GFP (Fig. S1A,B), as well as in 3D migration assays (Fig. S4B). Additionally, MDA-MB-231 cells expressing APPL1-AAA-GFP had similar MSD, persistence and directionality, compared with the control (Fig. S1C–E). Thus, APPL1 endosomal localization is important for its effect on cell migration.

Rab5 promotes cell migration and invasion, as well as integrin trafficking and adhesion turnover (Lanzetti and Di Fiore, 2008; Stupack and Torres, 2011; Valdembri et al., 2009). Since our results show that endosomal localization is important for APPL1-mediated migration, and APPL1 (but not an APPL1 mutant that cannot bind Rab5) affects integrin trafficking (Fig. 4E,F), we postulated that APPL1 affects cell migration through its interaction with Rab5. We generated a point mutation in the PH domain of APPL1 that reduces the interaction of APPL1 with Rab5 (N308D) (Zhu et al., 2007). The migration speed of cells expressing APPL1-N308D-GFP was not significantly different from cells expressing GFP in 2D (Fig. 5A,B) and 3D (Fig. S4C). Furthermore, APPL1-N308D-GFP expression in MDA-MB-231 cells had no effect on migration, whereas APPL1-GFP decreased migration speed and MSD, compared with GFP expression alone (Fig. S1A–C), suggesting that APPL1-mediated migration is dependent on its interaction with Rab5.

Next, we tested the ability of APPL1-GFP and/or point mutants of APPL1 to rescue the migration phenotype APPL1-depleted cells. HT1080 cells expressing NT gRNA or APPL1 gRNA#3 were transfected with GFP, APPL1-GFP, APPL1-AAA-GFP or APPL1-N308D-GFP, and migration assays were performed. As previously observed, APPL1 gRNA#3 cells increased migration speeds. However, expression of APPL1-GFP, but not APPL1-AAA-GFP or APPL1-N308D-GFP, in APPL1 gRNA#3 cells resulted in migration speeds similar to those in NT gRNA cells (Fig. 5C), suggesting that APPL1 expression can modulate migration, dependent on endosomal localization and interaction with Rab5.

To probe further the relationship between APPL1 and Rab5 in cell migration, APPL1-GFP and Rab5-mCherry and/or point mutants of each protein were co-expressed in HT1080 cells, and migration was assessed. Similarly to our previous results, cells co-expressing APPL1-GFP and mCherry (but not APPL1-N308D-GFP and mCherry) showed decreased migration speed compared with control cells (GFP and mCherry) (Fig. 5D). Cells expressing GFP and Rab5-mCherry exhibited increased migration speed, consistent with previous findings that Rab5 promotes migration (Stupack and Torres, 2011). Interestingly, co-expression of APPL1-GFP and Rab5-mCherry led to decreased migration compared with cells expressing Rab5 alone, indicating that APPL1-GFP expression suppresses Rab5-induced migration. Co-expression of APPL1-N308D-GFP with Rab5-mCherry did not abrogate Rab5-promoted migration, consistent with the reduced interaction of APPL1-N308D with Rab5. We generated a compensatory mutation in Rab5 described by Zhu et al. (2007) (L38R) that, when co-expressed with APPL1-N308D, re-establishes the interaction between APPL1 and Rab5. When APPL1-N308D-GFP and Rab5-L38R-mCherry were co-expressed, migration speeds were reduced to near control levels (Fig. 5D), suggesting that APPL1 reduces migration by antagonizing the ability of Rab5 to promote migration.

Next, we tested whether the interaction between APPL1 and Rab5 is important for the regulation of α5 integrin dynamics. Photoactivation of α5-PA-GFP at the leading edge of cells revealed that co-expressing either mCherry or APPL1-N308D-mCherry resulted in a more rapid loss of fluorescence than APPL1-mCherry, suggesting that the interaction of APPL1 with Rab5 is important for APPL1-mediated α5 integrin dynamics (Fig. S4D,E). In addition, we observed that APPL1 colocalizes with a subset of β1 integrin and Rab5 puncta (Fig. 5E). Since our results suggest that APPL1 inhibits...
α5 integrin dynamics (Fig. S4D,E) and α5β1 internalization in a Rab5-dependent manner (Fig. 4E,F), we hypothesized that APPL1 may inhibit the association between Rab5 and β1 integrin, thus slowing its trafficking and inhibiting migration. Indeed, overexpression of APPL1 reduces colocalization of Rab5 with β1 integrin (Fig. 5F,G), while APPL1 knockout increases colocalization between Rab5 and β1 integrin. **Fig. 5.** See next page for legend.
Fig. 5. APPL1 mediates migration through interaction with Rab5. (A) HT1080 cells expressing GFP, APPL1-GFP, APPL1-AAA-GFP or APPL1-N308D-GFP were plated on FN and migration assays were performed. Rose plots show individual track paths of representative cells from each condition. (B) Box plots showing quantification of migration speed from A. At least 50 cells were analyzed from each condition from three separate experiments (***P<0.0001, determined by one-way ANOVA followed by Tukey’s post hoc test). (C) HT1080 cells expressing APPL1 gRNA#3 or NT gRNA were transfected with GFP, APPL1-GFP, APPL1-AAA-GFP or APPL1-N308D-GFP, and migration assays were performed. Western blot shows APPL1 expression levels from each condition, with β-actin as a loading control. Box plot shows migration speeds from each condition. At least 35 cells total were analyzed from each condition from at least three separate experiments (**P<0.0001, compared with NT gRNA+GFP, determined by one-way ANOVA followed by Tukey’s post hoc test). (D) Box plot shows migration speeds for HT1080 cells that were co-transfected with GFP, APPL1-GFP or APPL1-N308D-GFP (N308D) and mCherry, Rab5-mCherry or Rab5-L38R-mCherry (L38R) and used in migration assays. –, expression of the tag only (GFP or mCherry); +, expression of APPL1-GFP or Rab5-mCherry. At least 44 cells total were analyzed from each condition from at least three separate experiments (**P<0.0001 compared with GFP+mCherry, ***P<0.0001 compared with GFP+mCherry, ***P<0.0001 compared with GFP+Rab5-mCherry, determined by one-way ANOVA followed by Tukey’s post hoc test). (E) HT1080 cells expressing APPL1-GFP immunostained for Rab5 and β1 integrin. Representative images are shown. Red/white boxes (single color/overlay, upper panels) indicate regions shown at higher magnification in bottom panels. Red/white arrowheads (single color/overlay) indicate colocalization between APPL1, Rab5 and β1 integrin. Scale bars: 15 μm (bottom). (F) HT1080 cells expressing GFP or APPL1-GFP immunostained for Rab5 and β1 integrin. Representative images are shown. Red/white boxes (single color/overlay) indicate regions shown at higher magnification. Red/white arrowheads (single color/overlay) indicate colocalization between Rab5 and β1 integrin. Scale bars: 15 μm (overlay) and 5 μm (bottom). (G) Quantification of percentage Rab5 colocalized with β1 integrin from F. Error bars represent s.e.m. from 57 cells from each condition from at least three separate experiments (**P<0.0001, determined by Student’s t-test).

Together, these results illustrate an important role for the interaction between APPL1 and Rab5 in regulating α5β1 integrin and cell migration.

APPL1 reduces the amount of active Rac in cells
Rab5 has been shown to promote Rac activation and thereby migration (Palamidessi et al., 2008). Since our results indicate that APPL1 negatively regulates the ability of Rab5 to promote migration, we hypothesized that APPL1 would inhibit Rac activity. To test this hypothesis, we examined the effect of APPL1 on cellular levels of activated Rac. HT1080 cells were plated on either FN or Coll and transfected with FLAG-Rac and either APPL1-GFP or GFP, and active Rac pulldown assays were performed. When APPL1-GFP-expressing cells were plated on FN, a 67% reduction in active Rac was observed, compared with cells expressing GFP (Fig. 6A,B). However, when cells were plated on Coll, no difference in the levels of active Rac was observed between cells expressing APPL1-GFP or GFP (Fig. 6C,D), which is consistent with our observations on APPL1-mediated migration. Rac activity was also assessed in APPL1-depleted cells. APPL1 gRNA#3 or NT gRNA cells were transfected with FLAG-Rac, and active Rac pulldown assays were performed. APPL1 gRNA#3 cells had increased levels of active Rac (~65%), compared with NT gRNA cells (Fig. 6E,F). Additionally, whereas expression of APPL1-GFP led to decreased Rac activity, APPL1-N308D-GFP had no effect on active Rac levels, compared with GFP-expressing cells (Fig. 6G,H). From these results, we conclude that APPL1 diminishes Rac activity in a Rab5-dependent manner.

APPL1 decreases activation of the Rac effector PAK
Since APPL1 decreased levels of activated Rac (Fig. 6), we hypothesized that APPL1 would also negatively regulate activity of the Rac effector PAK. phosphorylation of PAK at residue Thr423 is crucial for its activation (Zenke et al., 1999); therefore, the phosphorylation state of PAK can be used to assess PAK activity. We immunostained cells expressing APPL1-GFP or GFP with an antibody that recognizes PAK phosphorylated at Thr423 (pPAKThr423). When cells were plated on FN, the amount of pPAKThr423 (active PAK) was lower in cells expressing APPL1-GFP, compared with levels in the control (Fig. 7A,B). However, when cells were plated on Coll, APPL1-GFP expression had no effect on the levels of active PAK (Fig. 7C,D). APPL1-GFP expression had no effect on total PAK levels when plated either on either FN (Fig. S6A,B) or Coll (Fig. S6C,D). Consistent with our hypothesis, depletion of APPL1 resulted in a 1.4-fold increase in active PAK levels (Fig. 7E,F), but had no significant effect on total PAK levels (Fig. S6E,F). We confirmed our results biochemically, where APPL1 gRNA#3 cells led to a twofold increase in pPAK (Fig. 7G,H), but did not alter total PAK expression (Fig. 7G), compared with levels in NT gRNA cells. Together, these results implicate APPL1 in negatively regulating PAK activity.

If APPL1 acts upstream to negatively regulate PAK activation, then expression of a constitutively active form of PAK is predicted to abolish APPL1 regulation. To test this hypothesis, APPL1-GFP or GFP was co-expressed with a constitutively active variant of PAK [PAK-T423E (CA-PAK)] or control vector, and migration assays were performed. While APPL1-GFP-expressing cells migrated significantly slower than GFP-expressing cells when co-transfected with control vector, there was no significant difference in migration speed when APPL1-GFP was co-transfected with CA-PAK, compared with cells expressing GFP and CA-PAK (Fig. 7J), suggesting that CA-PAK abrogates the effect of APPL1 on cell migration. Similar results were observed for cells co-expressing APPL1-GFP and CA-PAK in 3D migration assays (Fig. S6G). These results support the hypothesis that APPL1 regulates migration by antagonizing PAK activity.

Because our results show that APPL1 mediates migration through its interaction with Rab5, a regulator of Rac activity, and APPL1 decreased Rac activity, we postulated that the negative effect of APPL1 on PAK would require Rab5 and endosomal localization. In both HT1080 and MDA-MB-231 cells, APPL1-GFP, but not APPL1-AAA-GFP or APPL1-N308D-GFP, decreased the amount of active PAK (Fig. 7K-N and Fig. S6H,I). Neither APPL1-GFP nor mutants of APPL1-GFP had any effect on total PAK levels in MDA-MB-231 (Fig. S6H,I) or HT1080 cells (Fig. S6K-N). These data point to a role for APPL1 in negatively regulating PAK activity through its interaction with Rab5.

PAK regulates cytoskeletal remodeling and focal adhesion dynamics at the leading edge of cells (Bokoch, 2003; Dharmawardhane et al., 1997); thus, we tested whether there were differences in PAK activity at the cell front in cells expressing APPL1-GFP, APPL1-GFP- and GFP-expressing cells were immunostained for pPAKThr423 and phallolidin. As shown in Fig. 7O, we observed lower levels of active PAK at the leading edge of the cell when APPL1-GFP was expressed. When we quantified PAK activity as a function of distance from the leading edge of cells (as determined by phallolidin staining), APPL1-GFP expression led to significantly lower levels of active PAK at the leading edge, and this difference was most pronounced at the very edge of the cell (Fig. 7P). Collectively, these results indicate that APPL1 diminishes PAK activity at the leading edge of migrating cells to impair cell migration.
DISCUSSION

Vesicular trafficking is known to contribute to cell migration processes, but the mechanisms are poorly understood. Here, we show that the endocytic adaptor protein APPL1 modulates migration on FN by altering α5β1 trafficking and Rac and PAK signaling through the interaction of APPL1 with Rab5 (see model in Fig. 8). Using a structure–function approach, we demonstrate that APPL1 interacts with Rab5 to inhibit Rab5-promoted migration. This inhibition results in higher surface levels of α5β1 integrin, because of decreased integrin endocytosis and increased integrin recycling. Furthermore, APPL1 decreases Rac activity, which, in turn, decreases PAK activity, particularly where PAK levels are most important at the leading edge. As a result, cell migration is impaired due to altered actin and adhesion dynamics.

APPL1 can act as the regulator for multiple pathways through its ability to engage in many protein–protein interactions and control trafficking. There is already evidence that APPL1 regulates crosstalk between multiple signaling pathways (Deepa and Dong, 2009; Rashid et al., 2009; Ryu et al., 2014). APPL1 mediates Akt signaling during cell migration, and our results point to a role for APPL1 in modulating Rac signaling as well. Both Akt and Rac signaling are crucial for migration (Ridley, 2015; Xue and Hemmings, 2013) and have been implicated in integrin trafficking (Jacquemet et al., 2013a,b). Our data indicate that most APPL1 functions in migration require APPL1 to bind to Rab5, including α5β1 internalization and activation of Rac and PAK. These data suggest a feedback cycle between integrin trafficking and cellular signaling that depends on trafficking through Rab5 compartments and is controlled by APPL1 (Fig. 8).
We note that APPL1 also interacts with Rab21, which is similar in structure to Rab5. Rab21 has been implicated in regulating integrin trafficking to promote cell migration (Pellinen et al., 2006). Therefore, we cannot rule out the possibility that the interaction between APPL1 and Rab21 contributes to APPL1-mediated effects on cell migration.
Our results here, together with our previous work (Broussard et al., 2012), indicate that APPL1 can be a negative regulator of cell migration, depending on the ECM substrate and the integrin repertoire (Figs 1 and 2). By contrast, other studies have reported that APPL1 is a positive regulator of migration (Tan et al., 2016, 2010). There are some key differences between these studies that may explain the contradictory results, including the use of embryonic mouse cells (Tan et al., 2010) versus human cancer cells (Fig. 1 and Fig. S1) (Broussard et al., 2012). Perhaps more importantly, the experiments performed by Tan et al. (2010) did not report the use of any ECM substrate coatings and instead induced migration with HGF. We did not explore the contribution of HGF to our phenotype.

Based on the substrate dependence of our finding (e.g. APPL1 regulates migration of cancer cells on FN but not Coll), we speculate that APPL1 might cause different phenotypes due to altered internalization of different migration-regulating receptors. Thus, whereas decreased internalization of α5β1 integrin leads to slower migration on FN, perhaps APPL1 also alters trafficking of the HGF receptor MET, which could lead to an increase in HGF-induced migration (Ménard et al., 2014; Muller et al., 2013). Tan et al. (2016) also implicated APPL2 in cell migration, acting redundantly with APPL1. However, although APPL2 is expressed in HT1080 cells, a 50% knockdown of APPL2 alone, or in APPL1-depleted cells, showed no effect on migration speed on FN (Fig. S1L). More studies should be performed in the future to reconcile these differences.

Our findings indicate that APPL1 regulates both integrin internalization and recycling. It is likely that APPL1 blocks internalization by inhibiting Rab5 from internalizing integrin, as expression of APPL1-N308D-GFP was unable to rescue the internalization phenotype in APPL1 gRNA#3 cells (Fig. 4E,F). Indeed, our results indicate that APPL1 affects colocalization of Rab5 with β1 integrin (Fig. 5F,G). Previous reports have implicated Rab5 and GIPC1 in promoting α5β1 integrin endocytosis from adhesion sites in endothelial cells, and it was suggested that APPL1 could act as a positive regulator in this process (Valdembri et al., 2009). In contrast, our results suggest that APPL1 is a negative regulator of integrin internalization, and this is dependent on its interaction with Rab5. Intriguingly, deletion of the PTB domain in APPL1 also failed to rescue control levels of integrin internalization in rescue experiments (Fig. 4E,F), consistent with the previously reported requirement for this domain to regulate cell migration and adhesion turnover (Broussard et al., 2012). As GIPC1 interacts with the C-terminus of the APPL1 PTB domain (Lin et al., 2006) and also binds α5 integrin (Tan and Mercurio, 2001), and APPL1 also interacts with Rab5, this is an interesting potential pathway for APPL1-mediated integrin trafficking. In addition, PTB domains of various proteins are known to bind NPxY motifs (Schlessinger and Lemmon, 2003) and β1 integrin has two NPxY motifs that regulate internalization and recycling (Böttcher et al., 2012; Margadant et al., 2012; Steinberg et al., 2012). Further study is required to determine if APPL1 acts through any of these mechanisms.

APPL1 has also been implicated in sorting proteins for recycling (Kalaidzidis et al., 2015), consistent with our observation that ANOVA followed by Tukey’s post hoc test (L,N)]. (J) Box plot for migration assays performed for cells co-transfected with APPL1-GFP or GFP and either constitutively active PAK (CA-PAK) or vector control. At least 25 cells total were analyzed from each condition from three separate experiments (**P<0.005, n.s., not significant, P=0.63, determined by Student’s t-test). (O) Cells expressing APPL1-GFP or GFP immunostained for F-actin (phalloidin, red) and p-PAKThr423 (pseudocolored for intensity). Red boxes in the second set of panels indicate regions shown at higher magnification in the third and fourth panels. White boxes represent a 5 μm (left) and 5 μm (right). (P) Linescan analysis performed at the leading edge of cells (identified by intense phalloidin staining) expressing APPL1-GFP or GFP. Quantification of the average fluorescence intensity is represented as a percentage of control (GFP at 0 μm). Error bars represent the standard error of the mean (s.e.m.) from three separate experiments. (**P<0.005, n.s., not significant, P=0.1, determined by Wilcoxon signed-rank test).

Fig. 8. Model for APPL1 regulation of α5β1 integrin trafficking and Rac activation in migrating cells. Dynamic recycling of α5β1 integrin through Rab5-positive endosomes (circle) promotes adhesion dynamics and cellular migration. In the presence of FN, APPL1 modulates α5β1 integrin internalization and recycling (left), and Rac and PAK activation (right) through negative interactions with Rab5 (Figs 4, 6 and 7). Recycling of activated Rac (Palamidessi et al., 2008) to the plasma membrane may promote cellular migration, potentially by activating PAK at the leading edge.

**Fig. 7. APPL1 decreases PAK activity.** (A,C,E,M) HT1080 cells expressing GFP or APPL1-GFP (A,C) and APPL1-AAA-GFP (K) or APPL1-N308D-GFP (M), APPL1 gRNA#3 or NT gRNA control cells (E) were plated on FN (A,E,K,M) or Coll (C) and immunostained for PAK phosphorylated at threonine 423 (p-PAKThr423). Representative images are pseudocolored for intensity. Scale bars: 15 μm. (B,D,F,L,N) Quantification of p-PAKThr423 levels from A,C,E,K and M, respectively, as a percentage of control. Error bars represent the s.e.m. from at least 58 cells total from each condition from three separate experiments [**P<0.05, **P<0.007, n.s., not significant, P=0.81, determined by Student’s t-test (B,D,F) or one-way ANOVA followed by Tukey’s post hoc test (L,N)]. (G) HT1080 cells expressing APPL1 gRNA#3 or NT gRNA (control) were lysed and western blots were performed for phosphorylated PAK (pPAK), total PAK, or β-actin (loading control). A representative image is shown. M, molecular weight marker. (H,I) Quantification of pPAK (normalized to total PAK levels) (H) or total PAK (I) levels from G as a percentage of control. Error bars represent the standard error of the mean (s.e.m.) from three separate experiments. (**P<0.005, n.s., not significant, P=0.3, determined by Student’s t-test). (J) Box plot for migration assays performed for cells co-transfected with APPL1-GFP or GFP and either constitutively active PAK (CA-PAK) or vector control. At least 25 cells total were analyzed from each condition from three separate experiments (**P<0.005, n.s., not significant, P=0.63, determined by Student’s t-test). (O) Cells expressing APPL1-GFP or GFP immunostained for F-actin (phalloidin, red) and p-PAKThr423 (pseudocolored for intensity). Red boxes in the second set of panels indicate regions shown at higher magnification in the third and fourth panels. White boxes represent a 5 μm (left) and 5 μm (right). (P) Linescan analysis performed at the leading edge of cells (identified by intense phalloidin staining) expressing APPL1-GFP or GFP. Quantification of the average fluorescence intensity is represented as a percentage of control (GFP at 0 μm). Error bars represent the s.e.m. from 45–50 cells total from each condition from three separate experiments (Z=−5.012, P<0.0001, determined by Wilcoxon signed-rank test).
APPL1 promotes recycling of α5β1 integrin (Fig. 4, Fig. S3). APPL1 colocalizes with some Arf6 compartments that contain the GTPase-activating protein (GAP) ARAP2 (ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 2). ARAP2 regulates transit of α5β1 integrin from APPL1 to early endosome antigen 1 (EEA1) compartments, and overexpression of ARAP2 promotes the formation of large adhesions (Chen et al., 2014), a phenotype similar to that of cells overexpressing APPL1 (Broussard et al., 2012). A future study should test whether the APPL1-dependent effects on integrin recycling are sensitive to ARAP2 levels.

Rab5 has been shown to promote Rac activation on endosomes (Diaz et al., 2014; Hagiwara et al., 2009; Palamidessi et al., 2008; Sandri et al., 2012; Torres et al., 2010). We found that APPL1 negatively regulates Rac (Fig. 6) and its effector, PAK (Fig. 7), depending on the interaction of APPL1 with Rab5. The regulation of Rac activation is linked to integrin trafficking (Sandri et al., 2012), as APPL1 decreased active Rac levels only when cells were plated on FN, and not on Coll (Fig. 6A–D). Since α5β1 integrin is internalized through caveolar-dependent endocytosis (Caswell and Norman, 2006; Shi and Sottile, 2008) and Rab5 is required for caveolin-dependent Rac activation (Diaz et al., 2014; Hagiwara et al., 2009), this could be a potential mechanism for APPL1-regulation of both Rac activity and integrin trafficking.

Interestingly, overexpression of APPL1 specifically decreases PAK activation at the leading edge of cells (Fig. 7O,P) where Rac activity is most important for cell migration (Broussard et al., 2008; Mayor and Etienne-Manneville, 2016; Noyal et al., 2006). It is currently unclear how PAK at the leading edge is regulated by APPL1-mediated changes in endosomal sorting. One possibility is that PAK is activated in Rab5-positive endosomes by Rac and recycled back to the cell surface; APPL1 might inhibit both the activation and the recycling. Alternatively, PAK could be activated in situ at the leading edge by recycled Rac (Palamidessi et al., 2008). As PAK is a key regulator of adhesion turnover (Delorme-Walker et al., 2011; Kiosses et al., 2002; Santiago-Medina et al., 2013; Webb et al., 2002), dynamic regulation through trafficking may ensure that it is present in the appropriate spatiotemporal manner (Disanza et al., 2009).

APPL1 is either deleted or mutated in multiple cancers (Cerami et al., 2012; Gao et al., 2013; Saleh et al., 2016), an observation consistent with our data suggesting that APPL1 is a negative regulator of cancer cell migration. In other cancers, APPL1 overexpression is accompanied by an increase in Rab5 expression (Bidkhori et al., 2013; Johnson et al., 2014; Zhai et al., 2016), implying that the ratio of these interacting proteins may be important for the cancer phenotype. In summary, our study supports a model where APPL1, in a FN-dependent manner, acts as a negative regulator of cancer cell migration through inhibition of Rab5-dependent processes, namely α5β1 integrin trafficking and activation of Rac and PAK.

MATERIALS AND METHODS

Plasmids

APPL1-GFP was prepared by cloning full-length APPL1 cDNA into pEGFP-C3 and mCherry-C3 vector as previously described (Broussard et al., 2012). APPL1-N308D-GFP and APPL1-N308D-mCherry were generated via site-directed mutagenesis of full-length APPL1-GFP and APPL1-mCherry, respectively, by the QuikChange II kit (Agilent manufacturer). Rab5-mCherry, respectively, was generated via site-directed mutagenesis of full-length APPL1 (Broussard et al., 2012). Rab5-mCherry was a kind gift from Jim Goldenring (Vanderbilt University, Nashville, TN). Rab5-L38R-mCherry was made by site-directed mutagenesis of full-length Rab5-mCherry using the following primers to mutate residue L38 to arginine: 5′-GGCCCTTTTCAAAAAACGGACGACTAAGTTGATTAGGTTGTTTGCATGCAGGTTTGGACAGCCC-3′ and 5′-ATTTATGATGCACAGGAATTCGCTAAGATCGGATTTATGATGCACAGGTTTGGACAGCCC-3′. Constitutively active PAK (PAKI-T423E) was generously provided by Jonathan Chernoff (Fox Chase Cancer Center, Philadelphia, PA). mCherry-paxillin was kindly provided by Steve Hanks (Vanderbilt University, Nashville, TN). α5-PA-GFP was a kind gift from Jim Norman (Beaton Institute for Cancer Research, Glasgow, Scotland, UK). THr-PA-mCherry was obtained from Addgene [plasmid #31948, deposited by Vladimir Verkhusha (Subach et al., 2009)]. siGENOME siRNA SMARTpool for APPL2 (M-016272-01-005) and non-targeting control (D-001206-14-05) were ordered from Dharmacon (Horizon Discovery). Clustered regularly interspaced short palindromic repeats (CRISPR) plasmids were obtained from ALSTEM (Richmond, CA). Three different guide RNAs were designed to target exon 2 of APPL1 and were inserted into PX459 vector. The guide RNA sequences are as follows: gRNA1, 5′-GCATCGGATTATGATGCAGC-3′; gRNA2, 5′-GAT-CATAGCGTTGATAACCTGG-3′; gRNA3, 5′-AGTTGTATCAAGCTATGCA-TTGAGC-3′. A non-targeting guide RNA (5′-GTTGAGTTTTGTAAGTCAGG-3′) in the PX459 vector was used as a control.

Antibodies and reagents

Primary antibodies used were as follows: mouse anti-β-actin (clone AC15, A5441, Sigma-Aldrich, St Louis, MO, 1:5000 for WB), rabbit anti-APPL1 (made using the peptides CSQSEESDLGEEGKKRESEA and CSQSESSLGDEGGKKRESEA, 21st Century Biochemicals, Malibar, MA), mouse anti-APPL2 (clone F2-2, 11140, Santa Cruz Biotechnology, Dallas, TX), mouse anti-integrin α5 (clone SNAKA51, MAB201, EMD Millipore, Billerica, MA), mouse anti-integrin α5 (clone 6F4, a kind gift from Rick Horwitz, Allen Institute for Cell Science, Seattle, WA), mouse anti-integrin β1 (clone 12G10, ab30394, Abcam, Cambridge, UK), mouse anti-integrin β1 (clone PS52, Abcam), rabbit anti-integrin β1 (AB1952, MilliporeSigma), rabbit anti-integrin β3 (ERP17507, Abcam), mouse anti-FLAG (clone M2, F1804, Sigma-Aldrich, 1:500 for WB), rabbit anti-phospho-T423PAK (#2601, Cell Signaling Technology, Danvers, MA), rabbit anti-phospho-S199-PAK (#2605, Cell Signaling Technology), rabbit anti-PAK1/2/3 (#2604, Cell Signaling Technology), rabbit anti-Rab5 (#3547, Cell Signaling Technology). Primary antibodies were diluted 1:500 for IF and 1:1000 for WB, unless noted otherwise. For function blocking experiments, mouse anti-integrin α5 (clone P1D6, MAB1956, Millipore) and mouse anti-integrin αV (clone 272-17E6, ab16821, Abcam) were used. Mouse IgG (#0107-01) was purchased from Southern Biotech (Birmingham, AL) and was used as a control in these experiments. For IF staining, Alexa Fluor 488 goat anti-mouse, 488 donkey anti-rabbit, 555 goat anti-rabbit, 647 goat anti-rabbit and 647 goat anti-mouse secondary antibodies were used at 1:1000 dilution (Thermo Fisher Scientific, Waltham, MA, for WB, Alexa Fluor® 680 donkey anti-mouse or goat anti-rabbit (Thermo Fisher Scientific) and IRDye800® donkey anti-mouse or goat anti-rabbit (Rockland, Inc., Limerick, PA), anti-mouse HRP-conjugated IgG (Rockland Immunoclochehicals) and anti-rabbit HRP-conjugated IgG (Promega) secondary antibodies were used. Fibronectin (FN; #80895), puromycin (#P7255) and primaquine bisphosphate (#160393) were purchased from Sigma-Aldrich. Rat-tail type I Collagen I (Col) was purchased from BD Biosciences (San Jose, CA). RGD and RGE peptides were from Bachem (Bubendorf, Switzerland).

Cell culture and transfection

HT1080 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Life Technology, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. MDA-MB-231 cells were maintained in Roswell Park Memorial Institute medium (RPMI) 1640 with 10% FBS and penicillin-streptomycin. Cells were transfected with Lipofectamine 2000 (Thermo Fisher Scientific) per the manufacturer’s instructions. For APPL2 knockdown, 50 nM of either APPL2 or non-targeting siRNA pool was transfected into HT1080 cells using DharmaFECT 1 transfection reagent (Horizon Discovery), following the manufacturer’s protocols. For migration assays and imaging experiments,
GFP-positive (or mCherry-positive) cells were chosen for analysis. For bulk biochemical assays, all cells were used.

3D cell culture
Rat tail ColI was mixed to a final concentration of 1.5 mg/ml in PBS and neutralized with NaOH (23 μl x volume of Coll solution) on ice. Cells were trypsinized and resuspended in DMEM (200,000 cells/ml); then, 500 μl of collagen solution was mixed with 500 μl of cell suspension and plated in 6-well cell culture dishes. Gels were allowed to solidify for 30 min at 37°C, and then 2 ml DMEM was added to the gels. Subsequently, gels were incubated at 37°C for 24 h to allow cells to attach and extend protrusions. 3D gels with embedded cells were used in migration assays.

Microscopy
A Quorum WaveFX spinning disk confocal system equipped with a Nikon Eclipse Ti microscope and a Hamamatsu ImageEM-C CD camera was used for imaging of immunofluorescence-stained coverslips, photoactivation experiments and 3D migration assays. An Apo TIRF 60x objective (NA 1.49) was used for imaging photoactivation experiments and immunofluorescence-stained coverslips. A Nikon 10x Ph1 ADL objective (NA 0.25) was used to image 3D cell migration assays. DAPI, Alexa Fluor 488, Alexa Fluor 555 and Alexa Fluor 647 were excited by the laser lines at 405 nm, 491 nm, 561 nm and 642 nm, respectively (Semrock, Rochester, NY). Emission filters for these fluorophores were 460/50, 525/50, 593/40 or 620/60, and 700/75, respectively (Semrock). TIRF microscopy and 2D migration assays were performed using an inverted Olympus IX71 microscope (Melville, NY) with a Retiga EXi CCD camera (QImaging, Surrey, BC). An Olympus UPlanFl N 10x objective (NA 0.30) was employed for cell migration assays. TIRF images were taken using an Olympus PlanApo 60x OTIRFM objective (NA 1.45) with a 543 nm laser line from a HeNe laser (Prairie Technologies, Middleton, WI). Images were acquired and analyzed using MetaMorph software (Molecular Devices, Sunnyvale, CA). Images from TIRF and photoactivation experiments were run through a 3x3 median filter using MetaMorph software to remove background.

Migration assays
Cell culture dishes were coated with either 2.5 μg/ml FN or 5 μg/ml rat tail Coll in PBS for 1 h at 37°C, after which cells were plated and permitted to adhere for 1 h at 37°C in cell culture medium. Cells were kept at 37°C in SFM4M Ab medium (HyClone, Logan, UT) supplemented with 2% FBS at pH 7.4 (imaging medium) during imaging using phase-contrast microscopy. Images were acquired at 5 min intervals for 6 h, using MetaMorph software. For 3D migration assays, the medium was changed to imaging medium after 24 h and cells were imaged. Migration was assessed for cells at least 100 μm deep into the gel to ensure that cells were in a 3D environment. For both 2D and 3D migration assays, cell movement was tracked from time-lapse images using MetaMorph, and migration speed was calculated by dividing the total distance moved in micrometers by the time. Persistence and directionality was quantified using an Excel macro described by Gorelik and Gautreau (2014). Wind rose plots were generated by transposing x,y coordinates of cell tracks to a common origin.

Immunofluorescence
For most experiments, cells were plated onto glass coverslips coated with 2.5 μg/ml FN or 5 μg/ml rat tail Coll and allowed to adhere for 1 h at 37°C. Cells were fixed using 4% paraformaldehyde supplemented with 0.12 M sucrase in PBS for 15 min at room temperature. Following fixation, cells were permeabilized with 0.2% (v/v) Triton X-100 for 3 min. Blocking was performed using 20% goat serum in PBS. Primary and secondary antibodies were diluted in 5% goat serum with 0.2% (v/v) Triton X-100 and were incubated with the cells at 4°C overnight or 45 min at room temperature, respectively. Following each antibody step, coverslips were washed with PBS extensively. Alexa Fluor 647 Phallolidin (#A22287, Thermo Fisher Scientific) co-staining was performed along with secondary antibodies. Coverslips were mounted on glass slides using Aqua Poly/Mount (Polysciences, Inc., Warrington, PA). For TIRF experiments, cells were plated on FN-coated glass bottom dishes, stained as described above, and kept submerged in PBS instead of mounted slides with Aqua Poly/Mount.

The average fluorescence intensity was quantified by dividing the background-corrected, integrated fluorescence intensity of individual cells by the cell unit area using MetaMorph software. The signal intensity was measured as the percent overlap between the two channels.

Photoactivation
Cells were co-transfected with α5-PA-GFP and either mCherry or APPL1-mCherry (or TfnR-PA-mCherry and either GFP or APPL1-GFP), plated on FN-coated, glass bottom dishes, and maintained in imaging media. A circular region of interest (ROI) at the edge of the cell was subjected to photoactivation using a 405 nm diode laser for 500 ms pulse with 100% laser power. For some experiments, APPL1 GRNA3 or NT gRNA cells were transfected with mCherry-paxillin, and adhesions were chosen as ROIs. Following photoactivation, images were taken every 10 s for 6 min using MetaMorph software. Images were taken before photoactivation to provide reference images. To analyze the kinetics of signal loss in the ROI, the average fluorescence intensity of the ROI was quantified for each time point and normalized to the average fluorescence intensity of the ROI from the first image post-photoactivation.

Antibody internalization and recycling assays
For the internalization assay, cells were plated on FN-coated coverslips and allowed to adhere for 1 h at 37°C. The cell surface was labeled by incubating with the appropriate antibody diluted 1:500 in PBS at 4°C for 45 min. Cells were washed with PBS, cell culture medium was added, and cells were returned to 37°C for 0–60 min to allow internalization. Cell surface labeling was removed by a wash with acetate acid, pH 2.5, on ice, for 5 min. Cells were fixed and stained with secondary antibodies as described above. The average fluorescence intensity for each time point was expressed as a percent of the maximum intensity measured per experiment (set to 100%), and the initial value was set to zero by subtracting the average fluorescence intensity at the 0 min time point from each value. In some experiments, cells were treated with 0.6 μM primaquine, a recycling inhibitor.

For visualizing internal levels of TfnR, cells were allowed to adhere for 1 h, and then 25 μl/ml pHodo Red transferrin conjugate (Thermo Fisher Scientific) was added to cell culture medium for the indicated time points. Coverslips were fixed and mounted as described for immunofluorescence.

For the recycling assay, cells were initially treated as described above for the internalization assay. Internalization was allowed to proceed for 30 min at 37°C in the presence of primaquine and cells were acid washed as described above. Cells were returned to 37°C in cell culture medium (without primaquine) for 0–20 min, acid washed and immunostained as described above. Recycling was quantified as a percentage of the original internal pool remaining at each time point, subtracted from the signal observed immediately following the first acid wash (where neither internalization nor recycling has occurred). The original internal pool was defined as the average fluorescence intensity at the 0 min time point (where internalization, but not recycling has occurred).

Western blot
The cells were lysed using RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) with protease inhibitor cocktail (Sigma-Aldrich). Protein concentration in the cell lysates was measured by the bicinchoninic acid (BCA) assay (Bio-Rad Laboratories, Hercules, CA) and 40 μg of each cell lysate was run in an SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was incubated with primary antibody diluted in 5% (v/v) non-fat dry milk in Tris-buffered saline, 0.1% (v/v) Tween 20 (TBS-T) at 4°C overnight, then incubated with the secondary antibody for 45 min at room temperature. Membranes were imaged using the Odyssey CLx imaging system (LI-COR Biosciences, Lincoln, NE). For phosphorylated PAK western blotting, cell lysates were then prepared with addition of PhosSTOP phosphatase inhibitor cocktail (MilliporeSigma) to the lysis buffer. HRP-
conjugated secondary antibodies were used and detected with SuperSignal West Femto maximum sensitivity substrate kit (Thermo Fisher Scientific) via an Amersham Imager 600 (GE Healthcare). Band intensities were measured using Image Studio Lite Software version 4 (LI-COR Biosciences) and normalized to a β-actin loading control.

**Biotinylation internalization and recycling assay**

For the internalization assay, HT1080 cells were transfected with the indicated constructs and after 6 h, the medium was changed to serum-free DMEM overnight. Per condition, 2.5×10^6 cells were plated in each well of a 6-well plate, grown to approximately 80% confluency, and then transfected with the indicated constructs. Cells were cooled with PBS for 45 min on ice to stop internalization. The cell surface was labeled with NHS-SS-Biotin (Thermo Fisher Scientific) at 4°C for 30 min. The biotinylation reaction was terminated by 5 min washes with 0.1 M glycine in PBS. For internalization, cells were washed PBS, cell culture medium with 0.6 μM primase was added, and cells were returned to 37°C for 0–60 min to allow internalization. Cell surface biotin was removed (except for total surface level condition) by two washes with 50 mM TCEP (Thermo Fisher Scientific) in NT buffer (150 mM NaCl, 1.0 mM EDTA, 0.2% BSA, 20 mM Tris-HCl, pH 8.6). Cells were lysed as described above and lysates were incubated with streptavidin agarose beads (Thermo Fisher Scientific) at 4°C for 30 min. The biotinylation reaction was terminated with RIPA buffer three times and protein was eluted with 5× sample buffer for 30 min, followed by western blot analysis.

For the recycling assay, cells were initially treated as described above for the internalization assay. One well in a 6-well plate was used for each condition. Internalization was allowed to proceed for 60 min at 37°C in the presence of primaquine and cells were washed with 50 mM TCEP as described above. Cells were returned to 37°C in cell culture medium (without primase) for 0–10 min, cell surface biotin was removed with 50 mM TCEP, and biotin was pulled down as described above. Recycling was quantified as a percentage of the original internal pool (the 0 min time point, where internalization, but not recycling has occurred) remaining at each time point.

**Active Rac pulldown assay**

Cells plated on cell culture dishes coated with 2.5 μg/ml FN or 5 μg/ml rat tail Coll were transfected with FLAG-Rac1 and indicated constructs. After 24 h, cells were lysed and assayed for active Rac as previously described (Bristow et al., 2009; Ren et al., 1999). In brief, lysates were incubated with glutathione Sepharose beads coated with GST-tagged p21 binding domain (PBD) from the Rac effector PAK to facilitate specific pull down of the active form of Rac. Whole-cell lysates (total Rac) and pulldown samples (active Rac) were loaded on the same gel and immunoblotted for FLAG and β-actin.

**Data analysis and statistics**

Statistical analysis was performed using SPSS software version 24. P-values were determined using either a Student’s t-test (if comparing two means), or a one-way ANOVA followed by Tukey’s post hoc test (if comparing more than two means), unless otherwise noted. In figures, *P<0.05, **P<0.005 and ***P<0.0005 (unless otherwise noted), and these were calculated as statistically significant. Bar graphs were generated using Microsoft Excel and presented as the mean±s.e.m. of at least three independent experiments. Box and whisker plots were generated using GraphPad Prism version 7.02 (GraphPad Software, La Jolla, CA). Boxes indicate ranges from 25 to 75th percentile, the line is the median, and the whiskers the minimum and maximum values.

**Acknowledgements**

We thank Alan Hall and Rick Horwitz for providing us with reagents. In addition, we thank Lan Hu for assistance in preparing cDNA constructs. We are very grateful to Kathy Friedman, Cristina Robinson and Begum Erdogan for their feedback and assistance with editing the manuscript.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**


**Funding**

This work was supported by National Institutes of Health (NIH) grants GM092914 and 5T1RR025524 to D.J.W. and R01GM117916 to A.M.W. and D.J.W. N.L.D. was supported by a Ruth L. Kirschstein National Research Service Award (NRSA) CA189710 from NIH. H.K. was supported by the Eunice Kennedy Shriver National Institute of Child Health and Human Development (EKS NICHD) of the NIH under Award# U54HD083211. Deposited in PMC for release after 12 months.

**Supplementary information**

Supplementary information available online at http://jcs.biologists.org/lookup/doi/10.1242/jcs.207019 supplementary

**References**


Journal of Cell Science


Supplementary Figures

A

GFP

150

APPL1-GFP

150

APPL1-AAA-GFP

150

APPL1-N308D-GFP

Supplementary Figure 1

B

Migration Speed (μm/min)

GFP

APPL1-GFP

APPL1-AAA-GFP

APPL1-N308D-GFP

C

MSD (μm²) x 10^4

0

60

120

180

Time interval (min)

n.s.

n.s.

n.s.

D

Persistence Index

GFP

APPL1-GFP

APPL1-AAA-GFP

APPL1-N308D-GFP

E

Direction autocorrelation

50

100

150

Time interval (min)

n.s.

n.s.

n.s.

F

Migration Speed (μm/min)

GFP

APPL1-GFP

C

MSD (μm²) x 10^4

0

60

120

180

Time interval (min)

n.s.

n.s.

n.s.

H

Persistence Index

GFP

APPL1-GFP

I

Direction autocorrelation

100

200

300

Time interval (min)

n.s.

n.s.

n.s.

J

NT

APPL2

M

siRNA

siRNA

IB: APPL2

IB: β-Actin

K

APPL2 expression

(% of Control)

NT

siRNA

APPL2

siRNA

L

Migration Speed (μm/min)

NT

APPL2

siRNA

siRNA

NT

APPL2

siRNA

M

RGE

RGD

GFP

APPL1-GFP

N

Migration Speed (μm/hr)

***

**

---

O

GFP

P1D6

APPL1-GFP

Supplementary Figure 2
Figure S1. **APPL1 regulates 2D and 3D cell migration.** (A) Migration of MDA-MB-231 cells expressing GFP, APPL1-GFP, APPL1-AAA-GFP, or APPL1-N308D-GFP was quantified. Rose plots show individual tracks of representative cells from each condition. (B) Box plot showing migration speeds for GFP-, APPL1-GFP-, APPL1-AAA-GFP-, and APPL1-N308D-GFP-expressing cells. 25-35 cells total were analyzed from each condition from at least three experiments (*, p<0.05, ***, p<0.0001, determined by one-way ANOVA followed by Tukey’s post hoc test). (C) MSD of MDA-MB-231 cells expressing GFP, APPL1-GFP, APPL1-AAA-GFP, or APPL1-N308D-GFP. Error bars represent s.e.m. from 25-35 cells total from each condition from at least three separate experiments (p<0.0001, comparing slopes of the lines using a likelihood ratio test). (D) Persistence index of MDA-MB-231 cells expressing GFP, APPL1-GFP, APPL1-AAA-GFP, or APPL1-N308D-GFP. Error bars represent s.e.m. from 25-35 cells total from each condition from at least three separate experiments (n.s., not significant, p=0.1, determined by one-way ANOVA). (E) Directional autocorrelation analysis of MDA-MB-231 cells expressing GFP, APPL1-GFP, APPL1-AAA-GFP, or APPL1-N308D-GFP. Error bars represent s.e.m. from 25-35 cells total from each condition from at least three separate experiments (p=0.992, 0.784, and 0.18, GFP compared to APPL1-GFP, APPL1-AAA-GFP, and APPL1-N308D-GFP, respectively, determined by mixed effects model to compare the curves, with controlling FDR at 0.1). (F) HT1080 cells were embedded in 1.5 mg/ml Coll matrices supplemented with 30µg/ml FN. Cells were allowed to adhere to matrices for 24 hours at 37°C, imaged with time-lapse microscopy, and migration speed was quantified for individual cells. Box plots show migration speed.
for cells expressing GFP or APPL1-GFP in 3D matrices. 24-31 cells total were analyzed from each condition from at least three experiments (***, p=0.0002, determined by Student’s t-test). (G) MSD of HT1080 cells expressing GFP or APPL1-GFP in 3D matrices. Error bars represent s.e.m. from 61-63 cells total from each condition from at least three separate experiments (p<0.0001, comparing slopes of the lines using a likelihood ratio test). (H) Persistence index of HT1080 cells expressing GFP or APPL1-GFP in 3D matrices. Error bars represent s.e.m. from 61-63 cells total from each condition from at least three separate experiments (n.s., not significant, p=0.17, determined by Student’s t-test). (I) Directional autocorrelation analysis of HT1080 cells expressing GFP or APPL1-GFP in 3D matrices. Error bars represent s.e.m. from 61-63 cells total from each condition from at least three separate experiments (p=0.974, determined by mixed effects model to compare the curves, with controlling false discovery rate (FDR) at 0.1). (J) Lysates derived from cells expressing APPL2 siRNA or non-targeting siRNA (control) were subjected to immunoblot analysis for levels of APPL2 and β-Actin (loading control). A representative image is shown. M, molecular weight marker; kDa, Kilodaltons. (K) Quantification of the endogenous levels of APPL2 in the blots described in (J), normalized to β-Actin. Error bars represent s.e.m. from three separate experiments (*p<0.05, determined by Student’s t-test). (L) Box plots showing migration speed on FN for HT1080 cells expressing APPL1 gRNA#3 or NT gRNA and either APPL2 siRNA or NT siRNA. 71-81 cells total were analyzed from each condition from three separate experiments (**p<0.0001, n.s., not significant, p>0.99, determined by one-way ANOVA). (M) Images of cells transfected with GFP or APPL1-GFP and treated with RGD peptide or RGE (control) show that this treatment did not
affect cell attachment. Scale bar = 20 µm. (N) Box plots showing migration speed for cells expressing GFP or APPL1-GFP and embedded in 3D matrices in the presence of RGD or RGE peptide (control). 12-18 cells total were analyzed from each condition from at least three experiments (***, p<0.0001, n.s., not significant, p=0.60, determined by Student’s t-test). (O) Images of cells transfected with GFP or APPL1-GFP and treated with α5 integrin function blocking antibody (P1D6) or control (IgG) show that this treatment did not affect cell attachment. Scale bar = 20 µm.
Supplementary Figure 2

**A**

![Graph showing TfnR-PA-mCherry Average Fluorescence Intensity in ROI (Relative to Control) over time.](image)

**B**

![Images of cells showing GFP and APPL1-GFP stained over time.](image)

**C**

![Graph showing Tfn-phrodo Red Average Fluorescence Intensity (% of Maximum Intensity) over time.](image)
Figure S2. APPL1 does not alter Transferrin Receptor trafficking. (A) HT1080 cells expressing TfnR-PA-mCherry and either GFP or APPL1-mCherry were subjected to live-cell photoactivation experiments. TfnR-PA-mCherry fluorescence was activated by stimulation with 405nm light within a ROI near the cell edge. Cells were imaged every 10 seconds for 30 seconds prior to photoactivation and for 6 minutes after photoactivation. Quantification of photoactivation experiments is shown as average fluorescence intensity, normalized to the amount of signal within the ROI at time=0. Error bars represent the s.e.m. 18 cells total were analyzed from each condition from three separate experiments. A nonparametric regression analysis was used to fit the curves (using natural cubic splines with three knots) and then compared the difference in the curves between two groups using the likelihood ratio test (not significant, p=0.026). (B) HT1080 cells expressing GFP or APPL1-GFP were plated on FN-coated coverslips and incubated with 25µl/ml Tfn-pHrodo Red at 37°C for 0-60 minutes. Cells were fixed and imaged to show internal levels of Tfn. Representative images are pseudocolored for intensity as indicated. Dotted lines demarcate the boundaries of the cell. Scale bar = 15µm. (C) Quantification of Tfn internalization assay from (B). Average fluorescence intensity is shown as a percent of the cell with the maximum intensity from each experiment. 58-60 cells total were analyzed from each condition from three separate experiments (n.s., not significant, p=0.70, 0.99, and 0.81 for the 20, 30, and 60 minute time points, respectively; determined by Student’s t-test).
Figure S3. APPL1 regulates α5β1 integrin internalization and recycling. (A, C, E) HT1080 cells expressing GFP or APPL1-GFP (A, C) or APPL1 gRNA#3 or NT gRNA (E) were surface labeled with a total β1 integrin antibody (clone P5D2) (A, E) or active β1 integrin antibody (clone 12G10) (C) at 4°C. Antibody internalization assays were performed as described in Methods. 0 minutes indicates cells that were surface labeled and acid washed, but not allowed to internalize integrin. Representative images are shown, pseudocolored to show intensity, where warm colors represent higher intensity and cool colors represent lower intensity. Dotted lines indicate cell boundaries. Scale bar = 15µm. (B, D, F) Quantification of β1 integrin internalization assay from (A, C, E), respectively. At least 58 cells (A, B), 40-60 cells (C, D), or 44-60 cells (E, F) total were analyzed from each condition from three separate experiments (*, p<0.05, **, p<0.005, ***, p<0.0005, determined by Student’s t-test). (G) HT1080 cells expressing either APPL1-GFP or GFP were used in β1 integrin internalization assays in the presence of primaquine or vehicle. Quantification of the average fluorescence intensity is shown as a percent normalized to the cell with the maximum intensity from each experiment. At least 46 cells total were analyzed from each condition from at least three separate experiments (*, p<0.05 compared to APPL1-GFP + Vehicle, ***, p<0.0001 compared to APPL1-GFP + Vehicle, determined by one-way ANOVA followed by Tukey’s post hoc test). (H) HT1080 cells expressing either APPL1-GFP or GFP were subjected to antibody recycling assays as described in Methods. (I) Quantification of the average fluorescence intensity from (H) is shown as a percent of the internal pool of integrin at time=0. 51-71 cells total were analyzed from each condition from three separate experiments (*, p<0.05, determined by Student’s t-test).
Figure S4. APPL1 requires endosomal localization and Rab5 interaction to regulate cell migration. (A) Top panels, GFP fluorescence in HT1080 cells expressing APPL1-GFP or APPL1-AAA-GFP. Scale bar = 15µm. Bottom panels, zoomed images of boxed regions from the top panels. Scale bar = 5µm. (B) Box plots showing migration speeds for cells expressing GFP, APPL1-GFP, or APPL1-AAA-GFP in 3D matrices. 16-31 cells total were analyzed from each condition from at least three experiments (**, p=0.0055, determined by one-way ANOVA followed by Tukey’s post hoc test). (C) Box plots showing migration speeds for cells expressing GFP, APPL1-GFP, or APPL1-N308D-GFP in 3D matrices. 15-23 cells total were analyzed from each condition from at least three experiments (***, p<0.0001, determined by one-way ANOVA followed by Tukey’s post hoc test). (D) HT1080 cells expressing α5-PA-GFP and either mCherry, APPL1-mCherry, or APPL1-N308D-mCherry were subjected to live-cell photoactivation experiments, as described in the legend to Figure 3. Images from selected time points are shown, pseudocolored to show intensity. Circle demarcates ROI used for photoactivation and subsequent average fluorescence intensity measurements. Dotted lines indicate the boundaries of the cell. Scale bar = 15 µm. (E) Quantification of photoactivation experiments as average fluorescence intensity, normalized to the amount of signal within the ROI in mCherry-expressing cells at time=0. Error bars represent the s.e.m. at each time point. 25-34 cells total were analyzed from each condition from three separate experiments. A nonparametric regression analysis was used to fit the curves (using natural cubic splines with three knots) and then compared the difference in the curves between three groups using the likelihood ratio test (p<0.001).
Figure S5. APPL1 depletion promotes association between Rab5 and β1 integrin.

(A) HT1080 cells expressing APPL1 gRNA#3 or NT gRNA were immunostained for Rab5 and β1 integrin. Representative images are shown. Red/white boxes (single color/overlay) indicate regions shown at higher magnification. Red/white arrowheads (single color/overlay) indicate colocalization between Rab5 and β1 integrin. Scale bar = 15 µm (overlay) and 5 µm (overlay zoomed images). (B) Quantification of % Rab5 colocalized with β1 integrin from (A). Error bars represent s.e.m. from 75 cells from each condition from at least three separate experiments (***, p<0.0001, determined by Student’s t-test).
Figure S6. APPL1 inhibits active PAK, but not total PAK, levels. (A, C, E) HT1080 cells expressing GFP or APPL1-GFP (A, C) or APPL1 gRNA#3 or non-targeting (NT) gRNA control cells (E) were plated on FN (A, E) or Coll (C) and immunostained for total PAK. Representative images are pseudocolored for intensity. Scale bar = 15 µm. (B, D, F) Quantification of total PAK levels from (A, C, E), respectively, shown as percent of control cells (GFP). Error bars represent the s.e.m. from >60 cells total from each condition from three separate experiments (n.s., not significant, p=0.10, 0.81, 0.09, respectively, determined by Student’s t-test). (G) Box plots showing migration speeds for cells in 3D matrices co-expressing either GFP or APPL1-GFP and either CA-PAK or control vector. 16-29 cells total were analyzed from each condition from at least three experiments (***, p<0.0001, n.s., not significant, p=0.65, determined by Student’s t-test). (H) MDA-MB-231 cells expressing GFP, APPL1-GFP, APPL1-AAA-GFP, or APPL1-N308D-GFP were immunostained for \textit{p-PAK}^{\text{Thr423}} or total PAK. Images are pseudocolored for intensity. Scale bar = 15 µm. (I) Quantification \textit{p-PAK}^{\text{Thr423}} levels from (H), shown as percent of control cells (GFP). Error bars represent the s.e.m. from 60-61 cells total from each condition from three separate experiments (**, p=0.002 determined by one-way ANOVA followed by Tukey’s post hoc test). (J) Quantification of total PAK levels from (H), shown as percent of control cells (GFP). Error bars represent the s.e.m. from 78-80 cells total from each condition from at least three separate experiments (n.s., not significant, p=0.64, determined by one-way ANOVA). (K, L) HT1080 cells expressing GFP, APPL1-GFP, or APPL1-AAA-GFP (K) or APPL1-N308D-GFP (L) were immunostained for total PAK.
Images are pseudocolored for intensity. Scale bar = 15 µm. (M, N) Quantification of total PAK levels from (K, L), respectively, shown as percent of control cells (GFP). Error bars represent the s.e.m. from 60-70 cells total from each condition from at least three separate experiments (n.s., not significant, p=0.77 and 0.84 from (M, N), respectively, determined by one-way ANOVA).
Supplementary Movies

**Movie S1. Cell migration of GFP-expressing cell.** HT1080 cells transfected with GFP were plated on FN and migration assays were performed. Images were taken every 5 minutes for six hours. Representative movie of a GFP-positive cell is shown. The blue dot represents the current position of a tracked cell, and the blue line represents the tracked path of the migrating cell. Scale bar = 50 µm.
Movie S2. Cell migration of APPL1-GFP-expressing cell. HT1080 cells transfected with APPL1-GFP were plated on FN and migration assays were performed. Images were taken every 5 minutes for six hours. Representative movie of a GFP-positive cell is shown. The blue dot represents the current position of a tracked cell, and the blue line represents the tracked path of the migrating cell. Scale bar = 50 µm.
Movie S3. 3D cell migration of GFP-expressing cell. HT1080 cells transfected with GFP were embedded in ColI gels and migration assays were performed. Images were taken every 5 minutes for six hours. Representative movie of a GFP-positive cell is shown. The blue dot represents the current position of a tracked cell, and the blue line represents the tracked path of the migrating cell. Scale bar = 20 µm.
Movie S4. 3D cell migration of APPL1-GFP-expressing cell. HT1080 cells transfected with APPL1-GFP were embedded in Coll gels and migration assays were performed. Images were taken every 5 minutes for six hours. Representative movie of a GFP-positive cell is shown. The green dot represents the current position of a tracked cell, and the green line represents the tracked path of the migrating cell. Scale bar = 20 µm.