 Novel function for AP-1B during cell migration

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ABSTRACT The epithelial cell-specific clathrin adaptor protein (AP)-1B has a well-established role in polarized sorting of cargos to the basolateral membrane. Here we show that β1 integrin was dependent on AP-1B and its coadapter, autosomal recessive hypercholesterolemia protein (ARH), for sorting to the basolateral membrane. We further demonstrate an unprecedented role for AP-1B at the basal plasma membrane during collective cell migration of epithelial sheets. During wound healing, expression of AP-1B (and ARH in AP-1B-positive cells) slowed epithelial-cell migration. We show that AP-1B colocalized with β1 integrin in focal adhesions during cell migration using confocal microscopy and total internal reflection fluorescence microscopy on fixed specimens. Further, AP-1B labeling in cell protrusions was distinct from labeling for the endocytic adaptor complex AP-2. Using stochastic optical reconstruction microscopy we identified numerous AP-1B-coated structures at or close to the basal plasma membrane in cell protrusions. In addition, immunoelectron microscopy showed AP-1B in coated pits and vesicles at the plasma membrane during cell migration. Lastly, quantitative real-time reverse transcription PCR analysis of human epithelial-derived cell lines revealed a loss of AP-1B expression in highly migratory metastatic cancer cells suggesting that AP-1B’s novel role at the basal plasma membrane during cell migration might be an anticancer mechanism.

INTRODUCTION Organ cavities are lined with columnar epithelial cells that organize apical domains luminally, whereas basolateral domains are contact-

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understood to be derived from renal proximal tubules that are naturally devoid of AP-1B expression (Roush et al., 1998; Schreiner et al., 2010). Since its discovery, LLC-PK1 cell lines with or without exogenous expression of µ1B to restore AP-1B function have been widely used to analyze polarized protein sorting (Fölsch, 2015a). Like other members of the family of heterotrimeric adaptor complexes such as AP-1, AP-2, AP-3, and AP-4, AP-1B directly recognizes cargos with YxxØ tyrosine-based motifs via µ1B and (D/E)xxxL(L/I) dileucine motifs, LL-motifs for short, through a shared surface between σ1 and γ adaptin (Ohno et al., 1999; Janvier et al., 2003; Doray et al., 2007).

Notably, although highly homologous, we and others found that AP-1A and AP-1B are functionally distinct. Whereas AP-1A is largely localized at the TGN and in early endosomes, AP-1B localizes and functions in REs of fully polarized epithelial cells (Fölsch et al., 2001; Gan et al., 2002; Gravotta et al., 2007). Although their localization as judged by immunofluorescence may appear mixed (Guo et al., 2013), the careful biochemical analysis showed that both complexes do not form mixed vesicle populations (Fölsch et al., 2003). Moreover, both AP-1A and AP-1B facilitated basolateral sorting from the TGN and REs, respectively, and thus have overlapping roles in the sorting of some, but not all, surface receptors (Fölsch et al., 1999; Kang and Fölsch, 2011; Gravotta et al., 2012; Caceres et al., 2019).

AP-1B selectively worked together with the coadapter autosomal recessive hypercholesterolemia protein (ARH) for sorting of an artificial cargo with FxNPxY signal (Kang and Fölsch, 2011). However, it remained to be shown if AP-1B and ARH would also cooperate in the sorting of endogenous cargos such as the focal adhesion molecule β1 integrin that contains FxNPxY sorting motifs (Moser et al., 2009). The interaction between ARH and AP-1B was mediated via the large β1 adaptin subunit (He et al., 2002; Mishra et al., 2002; Kang and Fölsch, 2011), and both AP-1A and AP-1B were pulled down by ARH in vitro; however, AP-1A did not cooperate with ARH in cells (see Figure 1A for a summary of known binding partners for AP-1B [Kang and Fölsch, 2011]). Most likely, the lipid environment present at the TGN that is enriched with phosphatidylinositol 4-phosphate (P(4)P; Wang et al., 2003) may not be amicable for ARH recruitment in contrast to REs (Mishra et al., 2002). Indeed, AP-1B expression in epithelial cells led to an accumulation of phosphatidylinositol 3,4,5-trisphosphate (P(3,4,5)P3) in REs, and in turn we also found that AP-1B localization in REs depended on P(3,4,5)P3 (Fields et al., 2010). This recruitment was mediated by µ1B, because mutations in a specific region of its cytosolic domain (R338N/N339S/V340E) that aligns with the P(4,5)P2 binding partner of µ2 (Collins et al., 2002) led to a loss of RE localization (Fields et al., 2010). In addition to P(3,4,5)P3, we identified Arf6 as important for AP-1B function (Shteyn et al., 2011). Arf6 precipitated AP-1A and AP-1B in vitro pull downs, and mutant Arf6 disrupted AP-1B sorting function in polarized cells (compare Figure 1A; Shteyn et al., 2011). Exogenous overexpression of Arf6 in LLC-PK1 cells grown in cell clusters induced ruffling. Importantly, only AP-1B but not AP-1A was recruited into these membrane ruffles (Shteyn et al., 2011). However, the role that AP-1B might play in membrane ruffles remained elusive.

It is generally accepted that whereas AP-2 facilitates clathrin-mediated endocytosis, all other heterotrimeric AP complexes facilitate sorting at the TGN or in endosomes (Hirst and Robinson, 1998; Boehm and Bonifacio, 2001; Nakatsu and Ohno, 2003). However, studies on ubiquitously expressed adaptor complexes are typically not performed in epithelial cells. Moreover, studies on AP-1B are typically performed in polarized cells grown in monolayers or cell clusters that are devoid of membrane ruffles. In this study, we demonstrate that in addition to its role in REs, AP-1B localizes to the plasma membrane in cell protrusions. This was not seen before, because LLC-PK1 cells typically don’t form membrane ruffles when grown in clusters or as monolayers for multiple days before immunofluorescence staining. However, LLC-PK1 cells start to ruffle and migrate when induced through Arf6 expression (Shteyn et al., 2011) or wounding of monolayers as presented in this study. Importantly, AP-1B localized in areas at the plasma membrane that were distinct from AP-2–positive areas. We further found that β1 integrin was an AP-1B and ARH cargo protein, and β1 integrin colocalized with AP-1B in cell protrusions during cell migration. Moreover, expression of AP-1B and ARH in AP-1B–positive cells slowed the speed of collective cell migration. Only AP-1B but not ARH expression was lost in highly metastatic cancer cells suggesting that AP-1B’s function during collective cell migration might be physiologically relevant.

RESULTS

Creation of LLC-PK1 cells stably expressing AP-1B-YFP

Because we found that AP-1B was recruited into cell protrusions facilitated by Arf6 (Shteyn et al., 2011), we wondered if AP-1B might localize to focal adhesions and play a role in cell migration. Focal adhesions are formed by integrin heterodimers, consisting of α and β integrin chain, that are attached to the extracellular matrix (Horton et al., 2016). They are necessary for forward movement, and integrin heterodimers that contain β1 integrin are well known for their role in generating speed during cell migration (Paul et al., 2015; Horton et al., 2016). Thus, to analyze a potential role for AP-1B in cell migration, we sought to test if 1) β1 integrin might be recognized as a cargo protein of AP-1B (and ARH), 2) if AP-1B expression might influence cell migration speed, and 3) if AP-1B might colocalize with β1 integrin during collective cell migration. To facilitate our studies, we created stable LLC-PK1 cell lines that expressed µ1A or µ1B with internal yellow fluorescent protein (YFP) tags as there are no suitable antibodies that would distinguish between µ1A/AP-1A and µ1B/AP-1B by immunofluorescence. The YFP-tags were placed between amino acids 230 and 231 as previously described for HA-tagged variants and guided by the crystal structure of µ2 (Owen and Evans, 1998; Fölsch et al., 2001). The newly created cell lines had modest expression levels as compared with endogenous µ1A, and comparable to the expression levels of the HA-tagged variants in LLC-PK1:γ1A-HA and LLC-PK1:µ1B-HA cell lines that were established earlier (Supplemental Figure S1A; Fölsch et al., 2001). Importantly, anti-GFP immunoprecipitations of µ1A-YFP and µ1B-YFP coprecipitated γ-adaptin indicating that they were incorporated into AP-1A-YFP and AP-1B-YFP complexes, respectively (Supplemental Figure S1B).

To test if AP-1B-YFP was incorporated into clathrin-coated vesicles, we seeded LLC-PK1:µ1B-YFP cells in 20-cm dishes. Monolayers were wounded with multiple scratches to create several wound edges throughout the surface of the plates and allowed to migrate for several hours before cells were homogenized and crude clathrin-coated vesicles were harvested by centrifugation in 1% Triton X-100 (Pears, 1982) to mimic wound healing assays used in this study. Pellets were resuspended in a sucrose buffer and subjected to immunosolubilations (Fölsch et al., 2003) with anti-GFP or anti-γ-adaptin antibodies followed by SDS–PAGE and Western blot (Figure S1, C and D). AP-1B-YFP was brought down in both immunoprecipitations. As expected, anti-GFP antibodies coprecipitated γ-adaptin and clathrin heavy chain (CHC), but not µ1A, consistent with our previous findings that AP-1A and AP-1B form biochemically distinct vesicle populations (Fölsch et al., 2003). Furthermore, anti-γ-adaptin
antibodies coprecipitated µ1B-YFP and endogenous µ1A as well as CHC. We conclude that AP-1B-YFP was incorporated into clathrin-coated vesicles that were distinct from AP-1A vesicles.

β1 integrin is an AP-1B (and ARH) cargo protein

β1 integrin has two FxNPxY motifs in its cytoplasmic tail (GENPIY and VVNPKY, Figure 1B; Moser et al., 2009) that have been reported to interact with endocytic coadaptors such as Dab2, numb, and ARH (Traub, 2009). Previously, we showed that ARH co-operated with AP-1B in REs to facilitate basolateral sorting of a model cargo protein with an FxNPxY motif (Kang and Fölsch, 2011). Therefore, we asked if β1 integrin depended on AP-1B (and ARH) for basolateral sorting in epithelia. To this end, we tested polarized sorting of β1 integrin in LLC-PK1 cells stably expressing µ1A (LLC-PK1::µ1A) or µ1B (LLC-PK1::µ1B) seeded on filter supports to allow for polarization. A basolateral-localized transmembrane protein is deemed an AP-1B cargo if it is sorted correctly to the basolateral membrane in the presence of AP-1B, but missorted in its absence (Fölsch et al., 1999; Fölsch, 2015a). Polarized cells were fixed and stained with antibodies directed against β1 integrin. Indeed, whereas β1 integrin was correctly sorted to the basolateral membrane in LLC-PK1::µ1B cells, β1 integrin was in part missorted to the apical membrane as well as endosomal puncta in LLC-PK1::µ1A cells (Figure 1D, compare schematic in Figure 1C). Note the disorganized appearance of LLC-PK1::µ1A cells that was also a feature of parental LLC-PK1 cells in the absence of AP-1B (Roush et al., 1998; motifs are annotated in red. (C) Schematic depicting apical, basal, and lateral membrane organization of polarized cells; black dots represent endosomes. (D–F) LLC-PK1::µ1B cells (D, left panels) and LLC-PK1::µ1A cells (D, right panels), LLC-PK1::µ1B-HA cells (E), and LLC-PK1::µ1B-YFP cells (F; cells with green signals express µ1B-YFP) were grown on filter supports for 4 d with gentle rocking from side-to-side for 3 d prior to fixation. After fixation, cells were stained for β1 integrin (magenta). GFP expression indicates shRNA presence. (D–G) Specimens were analyzed by confocal microscopy and representative cross-sections through assembled 3D galleries are shown. Bars are 10 µm.
FIGURE 2: AP-1B expression in epithelial cells slows down migration speeds. LLC-PK1, MDCK, and HBE cells were grown in Matrigel-coated MatTek dishes typically for 2 d. After wounding, cells were transferred to a Nikon BioStation for live imaging for up to 4 h. (A) Selected still images of LLC-PK1::µ1A (top panels) and LLC-PK1::µ1B (bottom panels) wounded monolayers at the beginning (0 h), 2 h, and 4 h after the start of data acquisition. Images of selected areas at the wound edge were taken every 15 min. Pixeled lines indicate wound edges at the beginning of live imaging. Bars are 50 µm. (B and C) Traveled path length (B) and distance (C) of migrating LLC-PK1 cells were determined using manual tracking of individual cells at the wound edge as described in Materials and Methods. Data represent mean values from three independent experiments. LLC-PK1::µ1A: cells from nine different areas (a total of 55 individual cells) were analyzed; LLC-PK1::µ1B: cells from 14 different areas (a total of 70 individual cells) were analyzed. (D) Area closure data...
Fölsch et al., 1999). Moreover, β1 integrin was also correctly sorted to the basolateral membrane in LLC-PK1:µ1B-HA and LLC-PK1:µ1B-YFP cells (Figure 1, E and F) indicating that the internal tags did not interfere with the sorting function of AP-1B. In addition, β1 integrin was missorted in Madin-Darby canine kidney (MDCK) cells depleted of µ1B (Gravotta et al., 2007). Therefore, basolateral sorting of β1 integrin depends on AP-1B.

To test if ARH cooperated with AP-1B in β1 integrin sorting, we depleted ARH or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as control in human bronchial epithelial (HBE) cells with short hairpin RNA (shRNA) vectors that also express GFP to indicate cells that are positive for shRNA expression using previously established protocols (Kang and Fölsch, 2011). In control HBE cells depleted of GAPDH, β1 integrin was correctly sorted to the basolateral membrane in addition to a few endosomal puncta. In contrast, β1 integrin was nonpolarized and partially missorted to the apical membrane after ARH depletion (Figure 1G). Note that we previously showed that depletion of ARH specifically affected basolateral sorting of FxNPxY-cargo proteins, but did not interfere with the basolateral sorting of cargos with YxxØ or LL-signals (Kang and Fölsch, 2011). We conclude that basolateral sorting of β1 integrin depends on both AP-1B and ARH. Thus, in addition to the previously identified artificial cargo protein LDLR-CT27, we here identify β1 integrin as an endogenous cargo for AP-1B and its coadapter ARH.

In summary, we generated cell lines stably expressing tagged forms of µ1B that are fully functional and we established the focal adhesion marker β1 integrin as an AP-1B (and ARH) cargo protein.

AP-1B expression slows down cell migration

β1 integrin is important for the generation of speed during cell migration (Paul et al., 2015). Because we found that β1 integrin is an AP-1B-dependent cargo protein, we wondered if AP-1B expression might influence the speed of collective cell migration. Thus, we seeded LLC-PK1:µ1A or LLC-PK1:µ1B cells on MatTek dishes coated with 1 mg/ml Matrigel. Cell monolayers were wounded and migration during wound healing was observed live in a Nikon BioStation for up to 4 h. Images of selected areas of the wound edge were taken every 15 min (Supplemental Videos figure2_AP1A.mov and figure2_AP1B.mov). Note the lack of dividing cells. Figure 2A shows still images at the beginning, after 2 h, and after 4 h of imaging. Using Nikon Elements software, individual cells at the wound edge were tracked manually to determine their traveled path length and distance (Figure 2, B and C). LLC-PK1:µ1A cells migrated with an average speed of 15.3 µm/h as opposed to 12.1 µm/h for LLC-PK1:µ1B cells, a reduction of 21%. This difference was statistically significant (P < 0.002) and was not a result of local directionality. When migration speeds were determined assessing the “straight” distance, LLC-PK1:µ1A cells traveled at a rate of 12.2 µm/h, whereas LLC-PK1:µ1B cells traveled 8.5 µm/h, a 31% reduction in migration speed (P < 0.003). Finally, we determined migration prowess by analyzing the total covered area of the migrating cells (Figure 2D). Whereas LLC-PK1:µ1A cells covered 5.2% of the total area of the imaged field per hour, LLC-PK1:µ1B cells covered only 3.3% of the total area per hour, a 37% reduction (P < 0.002). Although we rarely observed dividing cells during wound healing assays, growth rates were determined by counting cells at 0 and 48 h after seeding on coverglass coated with 1 mg/ml Matrigel. As expected, the times needed for cell numbers to double were comparable between LLC-PK1:µ1A cells (31 h) and LLC-PK1:µ1B cells (28 h) and therefore were not responsible for the observed differences in migration speeds (Figure 2E). Furthermore, we detected no differences in the arrangement of actin, microtubule, or keratin cytoskeleton at the leading edge between LLC-PK1:µ1A and LLC-PK1:µ1B cells (unpublished data, and compare actin staining in Figures 3 and 4).

In conclusion, using three different means of analyzing migration speeds, we found that the presence of AP-1B in LLC-PK1 cells slowed down cell migration in wound healing assays.

Importantly, the cell lines expressing HA or YFP-tagged µ1B/AP-1B also migrated at a slower pace as compared with the cell lines expressing the correspondingly tagged µ1A/AP-1A. Whereas LLC-PK1:µ1A-HA cells covered 3.6% of the total area per hour, LLC-PK1:µ1B-HA cells covered only 2.5% of the total area per hour, a 31% reduction (Figure 2F, P < 0.0003). Moreover, LLC-PK1:µ1A-YFP cells covered 3.5% of the observed area per hour compared with 2.5% coverage achieved by LLC-PK1:µ1B-YFP cells, a 29% reduction (Figure 2G, P < 0.006).

To make sure that the effect of AP-1B on cell migration was not specific to LLC-PK1 cells, we depleted µ1B expression in MDCK cells with shRNA using previously established protocols (Anderson et al., 2005; Fields et al., 2010) and determined migration speeds through area closure analysis (Figure 2H). Whereas mock-depleted MDCK cells covered 6.5% of the total area per hour, µ1B-depleted MDCK cells were able to cover 9% of the total area per hour, an increase by 38% (P < 0.0002). Thus, using gain-of-function and loss-of-function approaches, we found that AP-1B presence in epithelial cells decreased their migration speeds.

Previously, depleting ARH in cells without AP-1B expression led to a decrease in migration speed (Ezraty et al., 2009). Because we found that β1 integrin depended on both AP-1B and ARH for basolateral sorting (Figure 1), we next asked how ARH depletion would affect collective cell migration in AP-1B-positive cells. Therefore, we seeded HBE cells depleted of ARH or GAPDH as control on MatTek dishes coated with 1 mg/ml Matrigel and determined migration speeds in wound healing assays. Area closure analysis showed that...
whereas control HBE cells only covered 8.5% of the total area per hour, HBE cells depleted of ARH covered almost twice as much area with a speed of 15.4% of the total area per hour, an increase by 81% (Figure 2I, P < 0.0001). This increase in migration speed mimics the increase we saw after µ1B depletion in MDCK cells, but is in contrast to results obtained with AP–1B-negative cells (Ezratty et al., 2009) and may reflect ARH functions related to AP-1B.

In summary, both the expression of AP-1B and of ARH in AP–1B-positive epithelial cells slowed down the speed of migration in wound healing assays suggesting that AP-1B may regulate processes required for cell migration.

**AP-1B and β1 integrin colocalize in cell protrusions**

To investigate the localization of AP-1B during cell migration and test if AP-1B colocalized with β1 integrin, we wounded monolayers of LLC-PK1:µ1B-YFP cells grown on coverglass coated with 1 mg/ml Matrigel. Subsequently, cells were allowed to migrate as sheets for up to 6 h before fixation and containing for β1 integrin as well as the actin cytoskeleton and cell nuclei. Remarkably, confocal analysis revealed colocalization of AP-1B and β1 integrin at the edge of cell protrusions in front of actin arches (Figure 3A). To demonstrate this localization, we imaged 3D galleries throughout the specimens. Subsequently, the individual X-Y slices of the gallery were collapsed into one merged plane in a so-called “focused gallery projection” (see illustration in Figure 3A). Localization of AP-1B to β1 integrin foci was most evident in the coinciding appearance of peak intensities revealed through the line scan shown in Figure 3A. AP-1B-YFP and β1 integrin copelated at the cell edge in ~93% of analyzed cell protrusions. Note the absence of actin staining resulting in only baseline fluorescence in the area at the plasma membrane that was enriched in AP-1B and β1 integrin.

Next we sought to further investigate AP-1B localization in cell protrusions at higher resolution. To this end, we performed total internal reflection fluorescence (TIRF) microscopy on fixed and stained specimens. TIRF microscopy detects signals that are at or in close proximity to the basal plasma membrane in an evanescent field at the coverglass–specimen interface that is typically less than 100 nm (see illustration in Figure 3B; Mattheyses et al., 2010). LLC-PK1:µ1B-YFP cells were seeded on 1 mg/ml Matrigel-coated MatTek dishes. Monolayers were wounded and cells were allowed to migrate for several hours prior to fixation and containing for β1 integrin, CHC, and the actin cytoskeleton. Subsequently, specimens were maintained in buffer and imaged the same day as soon as possible. Wound edges were scanned and well-formed protrusions were imaged. In agreement with our confocal analysis, AP-1B-YFP staining was found at the very edge of cell protrusions colocalizing with β1 integrin as indicated by arrowheads in selected insets (Figure 3B).

In contrast to AP-1B-YFP labeling, we did not observe significant colocalization of AP-1A-YFP with β1 integrin in cell protrusions of migrating LLC-PK1:µ1A-YFP cells. Indeed, when analyzed with 3D confocal microscopy, we hardly detected any AP-1A-YFP label in migrating LLC-PK1::µ1A-YFP cells. Indeed, when analyzed with 3D confocal microscopy, we hardly detected any AP-1A-YFP label in migrating LLC-PK1::µ1A-YFP cells. Therefore, we employed an objective-based colocalization analysis that identified β1 integrin- and CHC-positive objects in AP–1–YFP-positive areas in well-formed cell protrusions as illustrated in Figure 4C (see Materials and Methods for further details). Data are expressed as objects in 100 YFP-positive areas to allow for comparison. Using this method, we detected 62.8 β1 integrin-positive objects within 100 AP–1B–YFP-positive areas, but only 26.2 β1 integrin-positive objects within 100 AP–1A–YFP-positive areas (Figure 4D, P < 0.003). In contrast and as expected, AP–1A–YFP and AP–1B–YFP showed about equal colocalization with CHC with 34 CHC-positive objects within 100 AP–1A–YFP-positive areas and 50.6 CHC-positive objects in 100 AP–1B–YFP-positive areas (Figure 4E). Lastly, 20.4% of AP–1B–YFP-positive areas contained both β1 integrin and CHC-positive objects in contrast to only 7.5% of AP–1A–YFP-positive areas containing both markers (Figure 4F, P < 0.02). Note the overlapping staining of both AP–1A and AP–1B with CHC is most likely underestimated, because a fully formed clathrin coat is expected to interfere with the ability of anti-GFP antibodies to label the YFP-tagged µ chains.

To summarize, AP–1B–YFP partially colocalized with β1 integrin in cell protrusions during collective cell migration. This colocalization was not observed for AP–1A–YFP.

**AP-1B does not colocalize with AP-2**

AP-2 is well known for its role in facilitating clathrin-mediated endocytosis of integrins (De Franceschi et al., 2016; Moreno-Layseca et al., 2019). Thus we wondered if AP–1B would colocalize with AP-2. To test this, we stained migrating LLC-PK1:µ1B-YFP cells with antibodies against α-adaptin, one of the large subunits of AP-2, as well as the actin cytoskeleton and analyzed the specimens with TIRF microscopy (Figure 5A). We found virtually no overlap between AP–1B–YFP and AP–2 staining. This is most obvious in the two-channel overlay of the insets. In contrast and as expected, µ1B-YFP showed colocalization with AP–1’s large γ-adaptin subunit to some degree (arrowheads in selected insets, Supplemental Figure 5D). Object-based colocalization analysis revealed 29.9 α-adaptin-positive and 68.4 γ-adaptin-positive objects within 100 µ1B–YFP-positive areas (Figure 5C, P < 0.0001). Note that colocalization between µ1B-YFP and γ-adaptin is most likely underestimated, because γ-adaptin staining labeled both AP–1B–YFP and endogenous AP–1A. In addition, whereas µ1B-YFP labels all AP–1B–YFP complexes, anti-γ-adaptin staining probably does not stain all complexes because CHC assembly most likely interferes sterically with anti-γ-adaptin antibodies reaching their epitopes.
FIGURE 3: AP-1B colocalizes with β1 integrin in cell protrusions. (A) LLC-PK1::µ1B-YFP cells were grown on Matrigel-coated coverglass for 2 d. Cells were wounded and fixed 6 h later. Specimens were stained for YFP (green), β1 integrin (red), actin cytoskeleton (blue), and nuclei (blue) and analyzed by confocal microscopy as depicted in the schematic. Representative collapsed images of acquired 3D galleries are shown. Stars (*) point to the edge of a cell protrusion. Arrow in the merged image indicates the line scan position used to generate the intensity profiles. The maximum intensity was 950, and the length of the line scan arrow was 10 µm. (B) LLC-PK1::µ1B-YFP cells were grown in Matrigel-coated MatTek dishes for 2 d, wounded, and fixed 4–6 h later. Cells were immunolabeled for YFP (green), β1 integrin (red), CHC (magenta), and the actin cytoskeleton (blue). Specimens were imaged by TIRF microscopy and representative images are shown. Rectangle in the merged image indicates area that was cropped for zoomed-in displays (insets). Arrowheads in the insets point to the β1 integrin-positive cell edges. Two-channel overlays were generated in Photoshop. Bars are 10 µm.
FIGURE 4: AP-1A does not colocalize with β1 integrin. (A) LLC-PK1::µ1A-YFP cells were grown on Matrigel-coated coverglass for 2 d. Cells were wounded and fixed 6 h later. Specimens were stained for YFP (green), β1 integrin (red), actin cytoskeleton (blue), and nuclei (blue), and analyzed by confocal microscopy. Representative collapsed images of acquired 3D galleries are shown. Stars (*) point to the edge of a cell protrusion. Arrow in the merged image indicates the line scan position used to generate the intensity profiles. The maximum intensity was 1050 with the 950 position marked, and the length of the line scan arrow was a little less than 10 µm with the 9.5 µm position marked in the profiles. The arrow in the line scan profiles points to the peak fluorescence of β1 integrin. Note the absence of both AP-1A and actin fluorescent staining at the leading edge. Bar is 10 µm. (B) LLC-PK1::µ1A-YFP cells were grown in Matrigel-coated MatTek dishes for 2 d, wounded, and fixed 4–6 h later. Cells were immunolabeled for YFP (green), β1 integrin (red), CHC (magenta), and the actin cytoskeleton (blue). Specimens were imaged by TIRF microscopy and representative images are shown. Bars are 10 µm. (C) Schematic...
Next we sought to confirm AP-1B localization in cell protrusions in LLC-PK1::μ1B-HA cells that express HA-tagged μ1B (Fölsch et al., 2001). Cells were again seeded and processed for TIRF analysis on fixed specimens. To this end, specimens were labeled for μ1B-HA/ AP-1B-HA, α-adaptin/AP-2, CHC, and the actin cytoskeleton. As seen for AP-1B-YFP, AP-1B-HA was found in cell protrusions in areas that were distinct from AP-2 labeling (Figure 5B, arrowheads 1 pointing to AP–2-positive foci, arrowheads 2 pointing to AP–1B– HA-positive foci; see Supplemental Figure S2E for a larger area of shown protrusion). Object-based colocalization analysis revealed only 15.3 AP–2-positive objects but 65.8 CHC-positive objects in 100 AP–1B–HA-positive areas (Figure 5D, P < 0.0001). In addition, we detected 48.4 CHC-positive objects per 100 AP–2–positive fields (Figure 5E). Note that colocalization between CHC and AP-1B as well as CHC and AP-2 at the leading edges seemed underwhelming. Although colocalization is most likely underestimated, because a fully formed clathrin coat is expected to interfere with adaptor complex labeling, a lack of profound colocalization between CHC and AP-2 in TIRF has been previously observed. This was in part due to a function for CHC in lamellipodia formation that was independent of adaptor complexes and membrane trafficking (Gautier et al., 2011).

In summary, AP-1B and AP-2 did not colocalize at the plasma membrane in cell protrusions during epithelial-sheet migration. As expected, both AP-1B and AP-2 colocalized to the same extent with CHC.

Super Resolution Microscopy of AP-1B in cell protrusions

To gather structural information of the AP-1B foci in cell protrusions, we performed stochastic optical reconstruction microscopy (STORM) that relies on detection of stochastic fluorophore “blinking” to obtain high-resolution images with a resolution limit of about 20 nm (van de Linde et al., 2011) and thus can theoretically distinguish between labeling of vesicles or endosomes as opposed to labeling of cytoskeletal elements as illustrated in Figure 6A. Furthermore, STORM is carried out in an evanescent field like TIRF microscopy and therefore mainly detects signals originating at or in close apposition to the basal plasma membrane. For STORM imaging, LLC-PK1::μ1B-HA cells as well as LLC-PK1::μ1A-HA cells as controls were grown in monolayers on MatTek dishes coated with 1 mg/ml Matrigel. Cells were wounded and allowed to migrate before being fixed and stained with anti-HA primary antibodies followed by Alexa 488-labeled secondary antibodies. Whereas we readily detected robust STORM signals in LLC-PK1::μ1B-HA cells, we detected virtually no STORM signals in LLC-PK1::μ1A-HA cells (Figure 6B, P < 0.0001), in agreement with our TIRF data showing that AP-1A-YFP fails to localize to the edge of cell protrusions (compare Figure 4B). As another internal, negative control, we detected no STORM signal in LLC-PK1 cells that were negative for μ1A-HA or μ1B-HA expression (unpublished data).

Figure 6C shows a representative cell protrusion of LLC-PK1::μ1B-HA cells analyzed with STORM. There are numerous AP–1B–HA-positive signals directly at the wound edge in addition to general labeling at the basal plasma membrane of the cell protrusion. These signals were scattered, suggesting AP-1B localization in vesicles as opposed to cytoskeletal structures. Indeed, when events were clustered (5 counts, 70-nm radius to mimic vesicles), many puncta remained (about 70% of events were identified in clusters for the cell shown). This was true for ~90% of analyzed cells.

Thus STORM analysis of LLC-PK1::μ1B-HA cells provides further evidence that AP-1B may be present in vesicles located close to or at the basal plasma membrane during cell migration.

AP-1B localizes to vesicles and pits at the plasma membrane

As a clathrin adaptor, AP-1B may facilitate the generation of clathrin-coated vesicles, and indeed immunosolubilation of AP-1B vesicles co-precipitated CHC (Fölsch et al., 2003, and Supplemental Figure S1C). Thus, to further clarify AP-1B’s localization with relation to the plasma membrane, we performed immunoelectron microscopy (immuno-EM) to investigate if AP-1B may be localized either in vesicles that invaginate from the plasma membrane or to pits that represent more flat structures that may or may not progress into forming vesicles (Wang et al., 2020). We chose LLC-PK1::μ1B-HA cells for these studies, because we previously had reliable results with immunogold labeling of AP-1B-HA (Fölsch et al., 2001, 2003). To enhance labeling efficiency and reduce steric hindrance of the gold-labeled secondary antibodies during pre-embedding labeling, we used nanogold (1.4 nm)-labeled secondary antibodies followed by a silver enhancement reaction during which silver precipitates around the gold particles. The precipitates present as amorphous, high-contrast structures in the EM images.

We readily found numerous AP-1B–HA-labeled structures that were in close apposition to the plasma membrane in migrating cells indicative of pits and forming vesicles at the plasma membrane (Figure 6D). A vesicle coated with AP-1B that is seemingly invaginating from the plasma membrane is indicated by arrow 1, whereas a pit coated with AP-1B is indicated by an arrow labeled 2 in Figure 6D.

Next we sought to determine labeling densities at the plasma membrane. To this end we took into consideration that AP-1B is a clathrin adaptor (Fölsch et al., 1999; Deborde et al., 2008), whose canonical recruitment of clathrin results in the formation of clathrin-coated vesicles (Fölsch et al., 2003, and Supplemental Figure S1C). The diameter of clathrin-coated vesicles typically ranges from about 75 to 130 nm (Pearse and Robinson, 1984; Kural et al., 2012). Thus, to determine labeling densities of AP-1B pertaining to invaginating vesicles and pits at the surrounding (lateral) plasma membrane, we determined total amounts of label as well as label within a narrow range of 50 and 100 nm from the surrounding membrane of whole cells at the wound edge imaged with a dark field detector. With a dark field detector, the silver precipitates appear white in a dark background allowing for easy detection (Supplemental Figure S3A). LLC-PK1::μ1A-HA cells were processed and imaged as control (Supplemental Figure S3B). Of the total signal, 3% of AP-1B-HA label was within 100 nm and 1.4% was within 50 nm of the surrounding membrane in comparison to only 1.6 and 0.5% of total AP-1A-HA label (Figure 6E, left graphs). Because cell shape varied (compare Supplemental Figure S3), we also determined label within 250 and 500 nm of the surrounding plasma membrane and calculated labeling densities in these areas. Within these areas, we again found that...
FIGURE 5: AP-1B does not colocalize with AP-2. LLC-PK1 cell lines stably expressing µ1B-YFP or µ1B-HA were grown in Matrigel-coated MatTek dishes for 2 d, wounded, and fixed 4-6 h later. (A) LLC-PK1::µ1B-YFP cells were immunolabeled for AP-2 (magenta) and the actin cytoskeleton (blue). AP-1B-YFP was imaged directly via the YFP signal. Specimens were imaged by TIRF microscopy and representative images are shown. Arrowheads in the insets point to AP–2-positive foci that are devoid of AP-1B-YFP labeling. Rectangle in the merged image indicates the area that was cropped for zoomed-in displays (insets). (B) LLC-PK1::µ1B-HA cells were immunolabeled for AP-2 (green), µ1B-HA/AP-1B-HA (red), CHC (magenta), and the actin cytoskeleton (blue). Specimens were imaged by TIRF microscopy and representative images are shown. Arrowhead 1 points to AP–2-positive foci and arrowhead 2 points to AP–1B–HA-positive foci. (A and B) Two-channel overlays were generated in Photoshop. Bars are 10 µm. (C–E) Object-based colocalization analyses were performed with Nikon Elements software as described in Materials and Methods (see also Figure 4C for a schematic illustration). (C) Results of object-based colocalization analysis of α-adaptin/AP–2-positive and γ-adaptin-positive objects in µ1B-YFP/AP–1B-YFP-positive areas. We analyzed 19 well-formed protrusions of LLC-PK1::µ1B-YFP cells from three independent experiments to score colocalization with α-adaptin and 26 well-formed protrusions of LLC-PK1::µ1B-YFP cells from three independent experiments to score colocalization with γ-adaptin. (D and E) Results of object-based...
the labeling density of AP-1B-HA at the lateral plasma membrane was significantly higher than the labeling density of AP-1A-HA (Figure 6E, right graphs). This confirms the immunofluorescence data acquired by either conventional confocal microscopy or TIRF imaging as shown in Figures 3 and 4. Importantly, we detected no label in cells that were negative for HA-tagged AP-1s (Supplemental Figure S3C). Note that with EM technology, it is impossible to discern how closely apposed labeling within the cell body is to the basal plasma membrane. Thus, although statistically significant, the amount of AP-1B-label at the plasma membrane is most likely underestimated, because we could only determine labeling density at the surrounding, lateral plasma membrane but not at the basal plasma membrane. This is in contrast to TIRF, where only label at or in close apposition to the basal plasma membrane is detected, allowing us to discern AP-1B label also at or close to the basal plasma membrane.

Taken together, our diverse imaging approaches ranging from conventional confocal microscopy, TIRF, and STORM imaging to immuno-EM all showed consistent labeling of AP-1B at or close to the plasma membrane with significantly less labeling of AP-1A in the same area. AP-1B labeling in close apposition to the plasma membrane in cell protrusions is a novel finding.

**AP-1B expression is lost in metastatic cancer cells**

Because AP-1B decreased cell migration speeds and localized close to or at the plasma membrane in cell protrusions during migration, we wondered if epithelial-derived, highly migratory cancer cells may have reduced levels of AP-1B. Therefore, we measured μ1B transcript levels by quantitative real-time reverse transcription PCR (qRT-PCR) in various human cell lines. Indeed, we found μ1B transcripts in normal breast epithelial cells MCF10A and 67NTER, as well as HBE and Caco-2 cells. However, we detected virtually no μ1B transcripts in the triple-negative breast cancer cell line MDA-MB-231 and the cervical cancer HeLa cell line (Figure 7A). This is in agreement with an earlier Northern blot analysis that showed expression of μ1B in Caco-2 cells and absence of μ1B in HeLa cells as well as fibroblast cell lines (Ohno et al., 1999). In contrast, ARH transcripts were found in all tested cell lines (Figure 7B).

Search for the μ1B gene (ap1m2) in the Cancer Dependency Map (depmap.org, ap1m2 search run in the second quarter of 2020 = 20Q2) revealed about 20 epithelial-derived carcinomas with missense or nonsense mutations in μ1B (Mutations Public 20Q2). Moreover, expression analysis of RNASeq data showed numerous epithelial-derived lines with lower to no expression of μ1B (Expression Public 20Q2). This is in line with earlier reports that more than 80% of kidney cancers arose in proximal kidney tubules (Holthofer et al., 1983; Gu et al., 1991; Martensson et al., 1995) that naturally lack μ1B expression (Scheiner et al., 2010). Moreover, μ1B expression was down-regulated in mouse models for colon cancer (Mimura et al., 2011). Thus, a lack of μ1B expression may be another hallmark of metastatic cancer.

**DISCUSSION**

Using wound healing assays we showed that AP-1B expression and ARH expression in AP-1B-positive epithelial cells slowed collective cell migration. We further established β1 integrin as a cargo protein that is sorted to the basolateral membrane by AP-1B and ARH. During collective cell migration, we found AP-1B colocalized with β1 integrin and clathrin at the plasma membrane in cell protrusions in areas that were not occupied by AP-2. STORM analysis was consistent with AP-1B’s presence in vesicles at or close to the basal plasma membrane. Indeed, EM analysis showed AP-1B localizing in coated pits and vesicles at the plasma membrane in migrating cells. Taken together, this study establishes localization of AP-1B at the plasma membrane in cell protrusions and implicates a regulatory role for AP-1B in cell migration. This novel function of AP-1B is not shared by AP-1A.

How does AP-1B influence migration speed? We found AP-1B colocalized with β1 integrin, and integrins regulate various cellular processes including cell spreading, formation of focal adhesions, and cell migration (Moreno-Layseca et al., 2019). Therefore, AP-1B’s role in migration is most likely linked to β1 integrin either in a structural or in a membrane trafficking function. Recently, CHC was implicated in Scar/Wave-mediated lamellipodia formation (Gautier et al., 2011). This role was uncoupled from CHC’s role in membrane trafficking and independent of clathrin light chain (Gautier et al., 2011). Moreover, AP-2 was shown to regulate adhesion in a 3D matrix independently from CHC by forming tubelike lattices around collagen fibers (Elkhaitib et al., 2017). Thus it might be that AP-1B has a similar structural function in cell migration and lamellipodia formation. Structural functions of AP-2 and AP-1B might further explain the relatively low level of colocalization between AP-2 and CHC as well as AP-1B and CHC in cell protrusions. Indeed, previous studies also noticed a lack of colocalization between AP-2 and CHC at cell edges (Gautier et al., 2011). However, positive roles in lamellipodia formation are typically associated with an increase in migration speed (Gautier et al., 2011; Paul et al., 2015; Moreno-Layseca et al., 2019) and thus cannot explain why AP-1B expression decreased migration speed.

It is further possible that AP-1B’s role in cell protrusions is to recycle and deliver β1 integrin to the leading edge (Figure 7C, possibility 1) Exocytosis. For example, family members of the monomeric clathrin adaptors Golgi-localized gamma-ear containing proteins (GGAs) have been implicated in integrin recycling. Indeed, GGA3 was shown to regulate β1 integrin trafficking to the cell periphery and depletion of GGA3 led to reduced cell migration (Ratcliffe et al., 2016). Furthermore, GGA2 together with its effector Rab13 were shown to promote recycling of β1 integrin and silencing of GGA2 or Rab13-reduced migration speed (Ratcliffe et al., 2016; Sahgal et al., 2019). Finally, GGA1 was found together with clathrin in “gyrating” structures at the basal membrane during migration that were suggested to be part of an endosomal recycling compartment and involved in rapid recycling (Zhao and Keen, 2008). Consisting with a role in recycling, depletion of GGA1 also reduced migration speeds (Ratcliffe et al., 2016). Thus, even though AP-1B may play its “normal” role in recycling during migration, this cannot explain why its depletion enhanced migration. Moreover, we found AP-1B label close to the cell periphery. Typically, one would expect vesicle coats to be shed close to the fusion site, indicating that this label may not represent exocytic vesicles. Indeed, using TIRF microscopy, exocytosis was observed all over the basal plasma membrane, but fusion of exocytic carriers was excluded within 1–2 μm from the cell edge.
FIGURE 6: AP-1B localizes in vesicles at the plasma membrane. (A) Schematic illustration of expected STORM results if vesicles are imaged in contrast to the cytoskeleton. Stochastic fluorophore blinking over many frames is reconstructed into a STORM image. (B and C) LLC-PK1:µ1B-HA cells were grown in Matrigel-coated MatTek dishes for 2 d. Cells were fixed 1.5 h after wounding and stained with anti-HA antibodies followed by Alexa 647-labeled secondary antibodies. STORM buffer was added and specimens were imaged using STORM followed by processing using Nikon Elements software as described in Materials and Methods. We recorded a total of 21 LLC-PK1:µ1B-HA cells in seven independent
 experiments. The percentage of cells at the wound edge that expressed AP-1B-HA and gave positive STORM signals was calculated per experiment and averaged as presented in the violin blot in B. No STORM signals were detected in LLC-PK1::µ1A-HA cells (four independent experiments). The cell shown in C had 19,639 out of 21,380 molecules identified as specific signals. 13,654 molecules were identified in clusters of at least five counts in a 70-nm radius. Arrowhead points to a clustered event at the edge of the cell protrusion. Bars are 10 µm. (D) LLC-PK1::µ1B-HA cells were wounded 2 d after seeding in Matrigel-coated MatTek dishes. After ~7 h, cells were fixed and processed for pre-embedding labeling with anti-HA primary and 1.4-nm gold-labeled secondary antibodies, followed by a silver enhancement reaction for 10 min, and processed for EM as described in Materials and Methods. Bright field images were acquired with the phase contrast bright field (TE) detector attached to the EM. Zoomed-in images in panel a were either cropped from the TIF file (a1) or directly imaged at higher resolution (a2). Bars as are indicated (500 nm for a; 100 nm for a2). Because a1 was cropped out of an existing TIF file, it has no scale bar. Numbered arrows point to the same regions in the individual images that are positive for AP-1B-HA labeling in coated vesicles (arrowhead 1) or pits (arrowhead 2) originating at the plasma membrane. (E) To determine labeling density at the plasma membrane (PM) EM images were analyzed using ImageJ software as described in Materials and Methods to determine total counts. We then counted individual labels within 50, 100, 250, and 500 nm from the PM. Data are expressed as percentages of label that occurred in close proximity (<50 and <100 nm) to the PM in relation to the total counts (left graph) or within a range of 500 or 250 nm from the PM (middle and right graphs). We analyzed a total of 34 AP-1A-HA and 25 AP-1B-HA expressing LLC-PK1 cells. To determine statistical significance, similar data sets between AP-1A-HA and AP-1B-HA expressing cells were compared (e.g., <50 nm/total AP-1A-HA vs. <50 nm/total AP-1B-HA and so forth). Error bars indicate SD. **P < 0.006; *P < 0.04.

lower the amount of mature focal adhesions that can form and thus lower migration speed. This would be consistent with AP-1B’s observed partial localization at the very edge of cell protrusion in a staining pattern that was largely distinct from actin labeling. Future studies are directed at distinguishing between these scenarios.

Recruitment of AP-1B to the plasma membrane in cell protrusions was probably facilitated by an enrichment in PI(3,4,5)P_3 found at these sites (Franca-Koh et al., 2007; Fields et al., 2010), and colocalization with β1 integrin was most likely bridged via ARH. Furthermore, we predict that Arf6 may also play a role in recruiting AP-1B into cell protrusions (Santy and Casanova, 2001; Shteyn et al., 2011). Currently, we are unable to easily and reliably costain for AP-1B and PI(3,4,5)P_3 and Arf6, as well as ARH during cell migration due to low transfection efficiencies. To this end, new stable cell lines would be required, which is beyond the scope of this study. Regardless, AP-1B did not colocalize with AP-2. Indeed, it is well known that membrane recruitment of AP-2 is strictly dependent on PI(4,5)P_2 and thus AP-2 should be absent from areas that are enriched in PI(3,4,5)P_3 (Collins et al., 2002). In contrast to AP-1B and AP-2, we found AP-1A largely absent from focal adhesions and the plasma membrane, most likely because AP-1A is normally recruited onto PI(4)P-positive membranes at the TGN and endosomes (Wang et al., 2003).

In fully polarized cells, AP-1B is not recruited to the plasma membrane at steady state although the basolateral domain is enriched in PI(3,4,5)P_3 (Gassama-Diagne et al., 2006; compare µ1B-YFP/AP-1B-YFP fluorescence in Figure 1F). Perhaps Arf6 is not activated at the basolateral membrane and therefore AP-1B may not be recruited. Indeed, the PH-domain of Grp1 that bound both PI(3,4,5)P_3 and activated Arf6 (Di Paolo and De Camilli, 2006; DiNitto et al., 2007) localized in RERs and membrane ruffles, but not at the lateral membranes (Fields et al., 2010). Moreover, whereas Arf6 facilitated endocytosis from the apical membrane (Altschuler et al., 1999), it played no role in endocytosis from the basolateral membrane of fully polarized MDCK cells (Boulant et al., 2011), consistent with an absence of active Arf6 at this site.

The fold change in migration speeds we observed in wound healing assays ranged between 20 and 40% both in gain-of-function (e.g., LLC-PK1 cell lines) or loss-of-function (e.g., depletion in MDCK cells) scenarios. These fold changes are in line with other migration studies done in epithelial cells. For example, depletion of E-cadherin in Caco-2 cells reduced migration speeds by approximately 50% (Hwang et al., 2012), and cleavage of the v-SNARE Vamp3 in MDCK cells stably expressing tetanus neurotoxin resulted in a reduction in velocity from about 17 µm/h to 8 µm/h (Proux-Gillardeaux et al., 2005). Vamp3 is needed for endosomal membrane trafficking, integrity of RERs, and AP-1B-facilitated exocytosis in epithelial cells (Galli et al., 1994; Daro et al., 1996; Fields et al., 2007).

E-cadherin is a major adherens junction protein with a highly conserved dleucine sorting signal (≤ LL-motif) for delivery to the basolateral membrane (Miranda et al., 2001). Although basolateral sorting does not depend on AP-1B per se (Miranda et al., 2001; Gravotta et al., 2007), AP-1B is necessary for efficient basolateral delivery of E-cadherin (Ling et al., 2007), and parental LLC-PK1 cells deliver less E-cadherin to the basolateral membrane than LLC-PK1:µ1B cells that express AP-1B (Ling et al., 2007). Based on these data, it is surprising that lack of AP-1B expression led to an increase in migration speed, because epithelial-sheet migration is positively correlated with E-cadherin levels at the basolateral membrane (Hwang et al., 2012): the more E-cadherin, the faster the speed. Thus, the positive effect that absence of AP-1B has on epithelial-cell migration may in part be countered by lower amounts of E-cadherin at the basolateral membrane. This may explain at least in part why depletion of ARH in AP-1B-expressing cells resulted in a greater change (~80% increase in speed) than depletion of AP-1B expression in MDCK cells. Unlike AP-1B, ARH only affects basolateral sorting of cargos with FxNPyX signals, but not cargos with Yxxδ and LL-based sorting signals (Kang and Fölsch, 2011) and is thus not necessary for delivery of E-cadherin to the basolateral membrane. Alternatively, ARH-independent functions of AP-1B in, for example, membrane recycling to the leading edge may lower the overall negative effect of AP-1B on migration and thus depletion of AP-1B may result in a lower net increase in speed as opposed to ARH depletion.

Curiously, depletion of ARH in AP-1B-negative cells resulted in slower cell migration (Ezratty et al., 2009). In fact, depletion of AP-2, ARH, and Dab-2 all inhibited focal adhesion turnover and resulted in decreased migration (Ezratty et al., 2009). Why then does ARH depletion in AP-1B-expressing cells enhance migration? It should be noted that unlike AP-2 that can cooperate with ARH, Dab-2, and numb (Traub, 2003), AP-1B can only cooperate with ARH (Kang and Fölsch, 2011). Therefore, ARH would be needed to target AP-1B to β1 integrin at the plasma membrane. The increase in migration speed after ARH depletion in AP-1B-positive cells may thus be a reflection of the inhibitory effect of AP-1B. This would also imply that ARH cooperates with AP-1B in controlling cell migration.

What are the physiological consequences of AP-1B expression in polarized epithelial cells? When we first described AP-1B, we noticed that re-expression of µ1B in LLC-PK1 cells had a profound effect on monolayer appearance. Whereas LLC-PK1:µ1B cell grew in monolayers, LLC-PK1:µ1A cells grew on top of each other (Fölsch et al., 1999). Indeed, µ1B knockout mice developed intestinal epithelial hyperplasia that was attributed in part to mislocalization of E-cadherin and activation of the β-catenin/Tcf4 complex (Hase et al., 2013). Curiously, β1 integrin, unlike other integrins, is localized not only to the basal but also to the lateral surface (compare Figure 1). Further, it was shown that β1 integrin facilitates cell–cell adhesion between keratinocytes and is needed for the invasive behavior of squamous cell carcinomas (Larjava et al., 1990; Brockbank et al., 2005). Thus at steady state, AP-1B may also aid in preventing abnormal growth by restricting β1 integrin at the basolateral membrane in addition to regulating E-cadherin (Hase et al., 2013). In the absence of AP-1B, β1 integrin is missorted to the apical membrane (compare Figure 1), most likely aided by galec-tin-3 (Höning et al., 2018). In highly metastatic cancer cells that lost expression of AP-1B (compare Figure 7), apically expressed β1 integrin could potentially help the survival of cells that are extruded apically from epithelial monolayers by promoting cell–cell adhesion at the apical membrane (Larjava et al., 1990). During cell migration, apically localized β1 integrin might facilitate intercalation of cells when wound edges close potentially leading to abnormal growth (Brockbank et al., 2005). Along these lines, histological studies suggested that > 80% of kidney cancers arose from proximal tubules that lack AP-1B expression (Holthofer et al., 1983; Gu et al., 1991; Martensson et al., 1995; Schreiner et al., 2010) implying AP-1B as an anticancer agent. This study continues shedding light on the underlying molecular mechanisms.

**MATERIALS AND METHODS**

**Antibodies and labeling dyes**

Rabbit anti-CHC (ab21679), rabbit anti-GFP (ab290), goat anti-GFP (ab6673), rabbit anti-α-adaptin (ab189995), and rabbit anti-γ-adaptin (ab220251), as well as CytoPainter Phalloidin–IFluor 405 (ab176752), were purchased from Abcam. Mouse anti-β1 integrin (MEM-101A) was purchased from Novus Biologicals, mouse anti-HA (16B12) from Covance, and mouse anti-γ-adaptin (100/3) from
Sigma. Hybridomas producing antibodies against LDL receptor (C7) were purchased from the American Type Culture Collection. Unlabeled, generic goat anti-rabbit IgG antibodies were purchased from Zymed. A polyclonal antibody cross-reacting between µ1A and µ1B was a kind gift from Linton Traub (University of Pittsburgh, Pittsburgh, PA).

Secondary antibodies labeled with Alexa dyes donkey anti-mouse Alexa 647, donkey anti-mouse Alexa 568, goat anti-mouse Alexa 488, donkey anti-goat Alexa 680, and donkey anti-goat Alexa 488 were purchased from Molecular Probes/Thermo Fisher Scientific. Donkey anti-mouse iRDiye 800CW and donkey anti-rabbit iR-Dye 680RD antibodies were purchased from Li-Cor. Donkey anti-rabbit Cy5 antibodies, and peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies were from Jackson ImmunoResearch. Goat anti-mouse antibodies were labeled with 1.4-nm colloidal gold as well as HQ Silver enhancement kit were from Nanoprobes. DAPI solution was purchased from BD Biosciences.

Cell culture
All cells were grown at 37°C in the presence of 5% CO₂. Their respective media were, in general, supplemented with 2 mM L-glutamine, 0.1 mg/ml penicillin/streptomycin, and 0.01 mg/ml ciprofloxacin. LLC-PK1 cells stably expressing µ1A, µ1B, µ1A-HA, µ1B-HA, µ1A-YFP, or µ1B-YFP were maintained in αMEM (5% fetal bovine serum [FBS]) containing 1 mg/ml geneticin, MDCK, and HBE cells were grown in MEM (5% FBS). HeLa cells were maintained in DMEM (10% FBS), MDA-MB-231 cells were maintained in αMEM (10% FBS), and Caco-2 cells were grown in DMEM (20% FBS) supplemented with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 10 µg/ml transferrin. MCF10A and 76NTER cells were maintained in DMEM/F12 medium (5% horse serum) supplemented with 20 ng/ml EGF, 0.5 mg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 µg/ml insulin, and 0.1 mg/ml penicillin/streptomycin. HBE and Caco-2 cells were maintained on coated surfaces. To prepare the coating solution, LHC basal medium was supplemented with 10 mg/ml bovine serum albumin (BSA), 3 mg/100 ml bovine collagen I, and 1 mg/100 ml fibronectin.

For wound healing assays, cells were seeded at densities ranging from 9 to 11 × 10^6 cells onto 22 × 22 mm coverglasses placed in 35-mm dishes (confocal analysis) or into MatTek dishes (35 mm with 14-mm microwell No. #1.5 coverglass for BioStation, fixed TIRF microscopy, STORM, and EM analysis) typically 2 d prior to wound healing assays such that cells formed monolayers without being overgrown. Coverglasses was acid washed and coated with 1 mg/ml Matrigel. Cell monolayers were scratched with a p200 tip to wound them, followed by two washes in growth media. Cells were typically fixed 4–6 h after wounding (1.5 h after wounding for STORM). Live imaging using the BioStation typically started 0.5 to 1 h after wounding.

To monitor polarized sorting of β1 integrin, we seeded 4 × 10⁵ cells on 12-mm filter supports (0.4-µm pore size; Corning) and cultured them for 4 d with changes of the medium in the basolateral chamber daily. LLC-PK1 cells were typically put on a rocker platform and rocked slowly and continuously for about 3 d prior to fixation. HBE cells were seeded on filter supports that were coated with 1 mg/ml Matrigel.

Cloning, retroviruses, and gene knockdown
The internal YFP-tags in µ1A and µ1B were introduced between amino acids 230 and 231 using PCR at the exact same position into which we previously introduced HA-tags (Fölsch et al., 2001). We first amplified N-terminal fragments (amino acids 1–230) of µ1A and µ1B with flanking EcoRI at the N-terminus and Xhol and Clal restriction sites at the C-terminus using the following primer pairs: 5'-GGGC GAATTCC ATG GCC TCG GCT TTC ATT-3' and 5'-GGGC ATCAGAT CTGGAG GGA TTT GCT GCT GCC AGT-3' for human µ1B and 5'-GGGC ATCAGAT CTGGAG GGA TTT GCT GCT CCC TCG GCCTGT-3' and 5'-GGGC GAATTCC ATG GCC AGC GCC GCT TAC GTA-3' for mouse µ1A. We used these fragments to exchange them with the N-terminal parts including the HA tag of our previous constructs through a simple cut-and-paste approach. We then amplified enhanced YFP using pEYFP-C1 as a template and the following primers: 5'-GGGC ATCAGAT ATGGT GAGCAAGGGCGAGGAG-3' and 5'-GGGC ATCAGAT CTTGTA CAGCTCGTCTCATGCC-3'. EYFP was introduced between Xhol and Clal sites in µ1A and µ1B using cut-and-paste technology. Constructs were confirmed through sequencing; no errors were found. The pcPB6 vector backbone now containing µ1A-YFP and µ1B-YFP were subsequently used to generate stable cell lines.

The shRNA Mir constructs in the lentiviral pGPi vector targeting human ARH (CGCGTGGCATTTAAGCACTTATAGTGAAGCCACAGA GTTATAATGTTTTAAGTGCAAGC) and human GAPDH were described previously (Nokes et al., 2008; Kang and Fölsch, 2011). The retroviral constructs in RVH1 specifically targeting canine µ1B (CCTCCGAATGTCGCAATGTGTTTCAAGAGAACCATTGGCCACCTGACTGCTTTTGGAAA) and scrambled control were as previously described (Anderson et al., 2005). HBE cells stably depleted of ARH or GAPDH as well as MDCK cells depleted of µ1B or scrambled control were generated by infecting HBE or MDCK cells with respective retroviruses exactly as previously described (Anderson et al., 2005; Pigati et al., 2013). Cells were maintained in growth media with 12 µg/ml puromycin. Depletion of ARH or µ1B mRNAs was monitored using RT-PCR.

RT-PCR and qRT-PCR
RNA was typically isolated from confluent monolayers of cells seeded in 10-cm plates using the direct-zol RNA miniprep kit from Zymo Research; 1.5 µg of purified RNA was used for reverse transcription using Superscript III enzyme (Invitrogen). Reverse-transcribed RNA was subsequently used for conventional RT-PCR or qRT-PCR using Taq polymerase. qRT-PCR was performed using the light Cycler 480 II real-time PCR machine from Roche and SYBR green detection.

(q)RT-PCR primers to amplify human ARH were 5'-ATCGTGGCTACAGCTAACGGC-3' and C-terminal primer 5'-GCCCTGCTGAGCCACAGAT-3', human µ1B was amplified using N-terminal primer 5'-TCCCTCCGGCAGCTCCTAGT-3' and C-terminal primer 5'-GGGCCACAAAGTAGAGGT-3', and human GAPDH was amplified using N-terminal primer 5'-ACAGTCGAGCCGCATCCTTT-3' and C-terminal primer 5'-CAATACGACAAAACTACCTGACT-3'. Canine ARH was amplified using N-terminal primer 5'-TGGCTCAGCATCGACAGGC-3' and C-terminal primer 5'-CTTGCGACATGCGCAATCT-3', canine µ1B was amplified using N-terminal primer 5'-TGGGTCAGTTTGAGACATCC-3' and C-terminal primer 5'-AGAAGCCGATCGACAATC-3'.

(q)RT-PCR (Figure 7) were run three times in triplicates on independently isolated RNA samples and analyzed using LightCycler 480 SW 1.5 software. Data were normalized to GAPDH RNA expression and mean values and SDs were determined. Mean values, SD, and n values were then used to calculate P values in unpaired Student's t tests using GraphPad QuickCalcs (GraphPad Software). Graphs were prepared using GraphPad Prism software.
BioStation imaging and data analysis

After wounding, cells were transferred to a Nikon BioStation IMQ equipped with a 20× objective (NA 0.8) and a high-sensitivity 1.3-megapixel cooled monochrome camera for brightfield imaging. Data acquisition typically started 30 min to 1 h after placing the specimens into the BioStation, dependent on how fast the system stabilized at 37°C and 5% CO2. Data were acquired with BioStation IM software and processed using Nikon Elements software. Manual single cell tracking throughout time frames to determine distance and path length traveled by individual cells in a monolayer at the wound edges was carried out with Nikon Elements AR3.2. To determine average speed, we first averaged individually imaged areas. Individual area data were subsequently averaged as presented in Figure 2, B and C. For area coverage analysis, BioStation nex files were converted to Nikon nci2 files and batch analysis was run using Nikon Elements 4.5. “Field Measurement” measured the biggest inverted area (i.e., the cell-free area) throughout the time frames (settings: 1. Cell: low pass filter, auto contrast, edge detection; 2. Only_biggest_invert cell: invert, fill holes, filter on object area). Frames were checked manually and were discarded if they could not be analyzed with this method. Area closure was determined as the percentage of the total area of the frame that became covered by migrating cells divided by the length of imaging (typically 1.5 to 3 h). To determine statistical significance, mean values and SDs were determined from the combined data points of at least three independent experiments. Mean values, SD, and n values were then used to calculate P-values in unpaired Student’s t tests using GraphPad QuickCalcs (GraphPad Software). Graphs were prepared using GraphPad Prism software. The percentage of changes were calculated by setting the control cell data at 100%.

Immunofluorescence staining and fluorescent imaging

Staining was performed essentially as previously described (Cook et al., 2011; Pigati et al., 2013). Briefly, after washing 3x with PBS2+ (phosphate-buffered saline [2.67 mM KCl, 1.47 mM KH2PO4] plus 0.901 mM Ca2+ and 0.493 mM Mg2+), cells were fixed with 3% PFA for 15 min (4% PFA for 20 min for fixed TIRF) at room temperature (RT), followed by a 5-min incubation in PBS+ at RT. If dealing with filter supports, filters were cut out as areas to be analyzed. On occasion, there were areas that overlapped partially or completely with the 488-positive areas. Frames were checked manually and were discarded if they could not be analyzed with this method. Area closure was determined as the percentage of the total area of the frame that became covered by migrating cells divided by the length of imaging (typically 1.5 to 3 h). To determine statistical significance, mean values and SDs were determined from the combined data points of at least three independent experiments. Mean values, SD, and n values were then used to calculate P-values in unpaired Student’s t tests using GraphPad QuickCalcs (GraphPad Software). Graphs were prepared using GraphPad Prism software. The percentage of changes were calculated by setting the control cell data at 100%.

Electron microscopy: pre-embedding immunolabeling, silver enhancement, and imaging

After three washes of the specimens in PBS2+ (PBS plus Ca2+ and Mg2+; see section on immunofluorescence staining), specimens were fixed with 8% PFA (EM grade) in 0.25 M HEPES buffer (pH about 7.1) for 1 h at RT. New fixative was then added followed by an overnight incubation at 4°C. The next day, specimens were washed 3 × 5 min each in PBS at RT with gentle shaking on a rocker. Specimens were then quenched for 15 min in 50 mM NH4Cl in PBS, followed by 3 washes in PBS, 5 min each, at RT with gentle shaking. Specimens were blocked with 2% goat serum, 0.1% BSA-c (from Auran), and 0.2% saponin in PBS for 1 h at RT. Anti-HA antibodies were diluted (1:75) in block solution and incubated with specimens for 1 h at RT followed by 4.5-h at 4°C. Subsequently, specimens were washed 3 × in PBS, 5 min each, at RT with gentle shaking. 1.4-nm gold-labeled secondary goat anti-mouse antibodies were diluted (1:50) in block solution and incubated with the specimens overnight at 4°C. The next day, specimens were washed 3 × in PBS,
5 min each, at RT with gentle shaking. After the last wash, specimens were incubated with 2% glutaraldehyde in PBS for 30 min at RT, followed by 3 washes in PBS, 5 min each, at RT with gentle shaking. Specimens were quenched for 5 min in 50 mM NH4Cl in deionized water at RT, followed by 2 washes in deionized water, 5 min each, at RT.

The subsequent silver enhancement reaction was carried out in a darkroom. The developer was prepared according to the manufacturer's instruction and 50-µl drops were added to the probes. Reactions were stopped after 10, 12.5 or 15 min by washing 6 × with deionized water, 2 min each, at RT. Samples were immediately post-fixed in 1% osmium tetroxide and 3% uranyl acetate, dehydrated in a series of ethanol washes and embedded in Epon 812 resin. The resin blocks were cured for 1 d at 56°C, and glass coverslips were removed from the blocks in liquid nitrogen. Ultrathin 70-nm sections were cut using a Leica UC7 Ultratome, deposited on copper grids and contrasted with Reynolds lead citrate and uranyl acetate. EM data were gathered with a Hitachi HD-2300A Dual EDS Cryo STEM operated at 200 kV utilizing the phase contrast bright field (TE) detector or the high angle annular dark field (HAADF) detector. Images were collected on Gatan Digital Micrograph with a DigiScan system. Images were processed and combined using Adobe Photoshop and Adobe Illustrator.

Statistical analysis was done with ImageJ software. To this end, labels in whole cell images were marked and counted. Next, cells were outlined, and the outside of the cells were cleared before converting the images into binary distance maps with units ranging from 0–255 where '0' marked the surrounding (lateral) plasma membrane and '255' marked the middle of the cells. Distances for individual spots were measured in the ROI manager. Subsequently, the amount of label within the 50, 100, 250 and 500 nm range away from the surrounding plasma membrane was determined using the known scale of images. Data are expressed as percentage of label in 50 or 100 nm with respect to the total counts or within a range of 250 or 500 nm. Mean values, SD, and n values were then used to calculate P values in unpaired Student's t tests using GraphPad QuickCalcs (GraphPad Software). Graphs were prepared using GraphPad Prism software.

Western blot and coimmunoprecipitations
For Western blot analysis of total cell lysates, cells were seeded at a density of 1:1 into six 20-cm dishes. Typically 2 d after seeding, cells were wounded by scratching the monolayers multiple times with a P1000 pipette tip and incubated for 1.5 h. Cells were then washed twice with PBS2+, and 2 ml buffer D (10 mM HEPES, 150 mM NaCl, 0.5 mM MgCl2, 1x protease inhibitors [mini tablets from Pierce], and 0.02 [wt/vol] NaN3) was added. Cells were scrapped off the plate, combined, and homogenized with a cell cracker followed by a clarifying spin in an Allegro D centrifuge (Beckman) at 4°C for 30 min at 5000 × g. Supernatants were harvested and adjusted with Triton X-100 to a final concentration of 1% and spun for 1 h in a MAX-E tabletop ultracentrifuge (Beckman) at 4°C and 100,000 × g (47,000 rpm, MLA80 rotor). Pellets were resuspended in 3 ml buffer D′ (buffer D plus 250 mM sucrose) and subjected to immunoprecipitations with goat anti-GFP antibodies (generic goat IgGs as control) or mouse anti-γ-adaptin antibodies (mouse hybridoma C7 antibodies as control) bound to protein G-Sepharose beads (Fisher Scientific). Samples were rotated end-over-end at 4°C for 2 h and washed 2x in buffer D′ and 1x in PBS. Immunoprecipitates were denatured in SDS sample buffer, vigorously shaken for 5 min at RT, boiled, and analyzed by SDS–PAGE and Western blot using fluorescently labeled secondary antibodies followed by analysis using a Li-Cor Odyssey Blot imager. Representative exposures were assembled and combined using Adobe Photoshop and Adobe Illustrator.

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