ALFY localizes to early endosomes and cellular protrusions to facilitate directional cell migration

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Summary statement

The autophagy-linked FYVE protein (ALFY) localizes to cell protrusions and early endosomes, and regulates directional cell migration and attachment by modification of integrin glycosylation.
Abstract

Cell migration is a complex process underlying physiological and pathological processes such as brain development and cancer metastasis. The autophagy-linked FYVE protein (ALFY), an autophagy adaptor protein known to promote clearance of protein aggregates, has been implicated in brain development and neural migration during cerebral cortical neurogenesis in mice. However, a specific role of ALFY in cell motility and extracellular matrix adhesion during migration has not been investigated. Here we reveal a novel role for ALFY in the endocytic pathway and in cell migration. We show that ALFY localizes to RAB5 and EEA1 positive early endosomes in a PtdIns(3)P dependent manner and is highly enriched in cellular protrusions at the leading and lagging edge of migrating cells. We find that cells lacking ALFY have reduced attachment and altered protein levels and glycosylation of integrins, resulting in the inability to form a proper leading edge and loss of directional cell motility.

Introduction

ALFY/WDFY3 is a large evolutionary conserved protein known to promote clearance of protein aggregates through its interaction with autophagy components and phosphatidylinositol-3-phosphate (PtdIns(3)P) (Simonsen et al., 2004, Lystad et al., 2014, Clausen et al., 2010, Filimonenko et al., 2010, Han et al., 2015, Fox et al., 2019). Depletion of ALFY leads to accumulation of ubiquitin positive aggregates (Filimonenko et al., 2010, Finley et al., 2003), and its heterozygous depletion significantly accelerates age of onset and pathogenesis in a mouse model of Huntington’s disease (Fox et al., 2019, Dragich et al., 2016). Alfy knock-out (KO) mice die a few hours after birth and are characterized by loss and disorganization of interhemispheric axonal tracts throughout the brain, suggesting that Alfy is involved in neural migration during cerebral cortical neurogenesis (Dragich et al., 2016, Kadir et al., 2016, Orosco et al., 2014).

Cell migration involves cell polarization to develop a leading and a trailing end, followed by recruitment of the actin cytoskeleton and formation of cell protrusions and focal adhesions (FAs) at the leading edge. FAs are large complexes consisting of multiple scaffolding and signalling proteins, such as integrins, paxillin and vinculin, which link the extracellular matrix to intracellular signaling pathways, leading to actin remodeling and traction required for forward cellular movement (Winograd-Katz et al., 2014). Dynamic assembly and disassembly of FAs is important for productive displacement of the cell body as the cell moves (Kenific and Debnath, 2016). During cell migration, integrins, the transmembrane proteins of FAs, are internalized into RAB5-positive
early endosomes, from where they can be targeted for lysosomal degradation or recycled to the plasma membrane of the leading edge, via RAB11-positive recycling endosomes, to facilitate another round of cell motility (Maritzen et al., 2015, Shafaq-Zadah et al., 2016, Nader et al., 2016).

Here, we report a novel role for ALFY in directional cell migration. We show that ALFY is highly enriched in cellular protrusions and early endosomes and that it colocalizes with integrin-α5 and regulates protein levels and glycosylation of several integrins, implying it regulates integrin trafficking during directional cell migration.

**Results and Discussion**

**ALFY localizes to cellular protrusions**

ALFY contains several C-terminal domains, including a PH-BEACH-domain with unknown function, five WD repeats, an LC3-interacting region (LIR) that binds to GABARAP proteins and a PtdIns(3)P binding FYVE-domain (Simonsen et al., 2004, Lystad et al., 2014) (Fig. 1A). The large size of ALFY (3526 amino acids, 68 exons) has made it difficult to clone and express the full-length protein and previous studies have therefore been based on immunostaining of fixed cells with an anti-ALFY antibody or expression of deletion mutants (Clausen et al., 2010, Simonsen et al., 2004). We have now successfully cloned and expressed full length ALFY, tagged with enhanced green fluorescent protein (EGFP) or tandem-dimer NeonGreen (tdNG), in HeLa and U2OS T-Rex Flp-In cells, respectively (Fig. S1A-B). Live-cell imaging of both cell lines shows ALFY localization to small dynamic vesicle-like structures distributed throughout the cytoplasm, with enrichment in cellular protrusions and close to the basal plasma membrane (Fig. 1B, S1C). Interestingly, the ALFY-positive structures were actively redistributed to the advancing leading edge and retracting lagging edge of migrating cells (Fig. 1C), indicating a role for ALFY during cell migration.

To further study the localization and function of wild-type (WT) and mutant ALFY proteins, ALFY was depleted in HeLa T-Rex Flp-In cells (HeLa ALFYKO) with two different guide RNAs (Fig. S1D), as confirmed by western blot (Fig. 1D, S1E) and immunoprecipitation with several anti-ALFY antibodies (Fig. S1F). Several KO clones were selected for further studies (ALFYKO1-1, ALFYKO2-6, ALFYKO2-9 and ALFYKO2-11) and for generation of rescue cell lines with inducible expression of EGFP-ALFY WT, LIR or FYVE domain mutatants (Fig. 1E, 4B). Analogous to EGFP-ALFY, both EGFP-ALFY LIRmut and FYVEmut localized to intracellular structures and to cellular protrusions (Fig. 1F, G), indicating that localization of ALFY to protrusions occurs
independently of its binding to GABARAP proteins and PtdIn(3)P, respectively (Simonsen et al., 2004, Lystad et al., 2014). Likewise, localization of EGFP-ALFY to protrusions was unaffected in cells treated with the VPS34 inhibitor (VPS34IN1) (Fig. 1F, G). In contrast, ALFY mutants lacking either the WD40-FYVE domains (EGFP-ALFY ΔWD40-FYVE) or the PH-BEACH domains (EGFP-ALFY ΔPH-BEACH) did not show an enrichment in cellular protrusions (Fig. 1F, G), suggesting a role for its C-terminal part in targeting to cellular protrusions.

ALFY localizes to early endosomes in a PtdIns(3)P dependent manner

The nature of the EGFP-ALFY-positive structures was analyzed by co-immunostaining for different cytoplasmic markers. Surprisingly, EGFP-ALFY colocalized extensively with the early endosome markers EEA1 and RAB5 (Fig. 2A-B, Movie 1), while dynamic “kiss and run” interactions were detected with the late endosome marker RAB7 or the recycling endosome marker Rab11 (Fig. S1G, S1H, Movie 2-3). We noticed during live cell imaging that the subcellular localization of EGFP-ALFY is highly sensitivity to temperature, as its localization to cellular protrusions was lost upon washing cells with cold PBS for 10 minutes at room temperature (Fig. S1I). Given that many intracellular events, including endocytosis and secretion, are inhibited at low temperature (Saraste et al., 1986, Tomoda et al., 1989), and the weak binding of the ALFY FYVE domain to PtdIns(3)P containing membranes (Reinhart et al., 2021), it is likely that recruitment of ALFY to early endosomes might be activity dependent.

As neither the LIR nor the FYVE domain of ALFY are essential for its localization to protrusions (Fig. 1F, G), we asked whether these motifs are required for recruitment of ALFY to early endosomes. HeLa ALFYKO1-1 cells expressing either EGFP-ALFY, -LIRmut or -FYVEmut were transfected with a constitutively active GTPase-deficient RAB5 mutant (myc-RAB5Q79L) that generates enlarged early endosomes. Both EGFP-ALFY and -LIRmut were efficiently recruited to the myc-RAB5Q79L vesicles, while recruitment of EGFP-ALFY-FYVEmut was strongly reduced (Fig. 2C), indicating that recruitment of ALFY to early endosomes requires its FYVE domain, but is independent of its binding to GABARAP. The EGFP-ALFY-FYVEmut did however localize to small RAB5 negative structures (Fig. 2C, S2B), in line with the presence of EGFP-ALFY positive structures in cells treated with VPS34IN1 (Fig. 1F).
As reported previously (Simonsen et al., 2004, Lystad et al., 2014), we observed some colocalization of EGFP-ALFY with the autophagy markers mScarlet-I-LC3B (Fig. 2D) and -GABARAP (Fig. 2E), while full length ALFY failed to colocalize with the autophagy receptor mScarlet-I-SQSTM1 under basal conditions (Fig. S2A).

To elucidate a possible endosomal function of ALFY, we analyzed recycling of transferrin (Tfn) and degradation of the Tfn-receptor (TfR) as well as degradation of epidermal growth factor (EGF)-receptor (EGFR) and rhodamine-EGF in WT and ALFY\textsuperscript{KO1-1}. While Tfn-recycling and TfR-degradation was unaffected in ALFY\textsuperscript{KO1-1} cells (Fig. S2C-E), the level of EGFR and rhodamine-EGF was reduced in ALFY\textsuperscript{KO1-1} cells compared to WT (Fig. S2F-J), suggesting that ALFY regulates cargo sorting of EGFR from early endosomes to lysosomes.

Taken together, we show that EGFP-ALFY localizes to early endosomes in a PtdIns(3)P dependent manner, but that it also is recruited to cytoplasmic structures independently of PtdIns(3)P.

**ALFY is required for directional cell migration**

The localization of ALFY to the leading and lagging edges of migrating cells, as well as its role in neural migration during cerebral cortical neurogenesis, prompted us to investigate a role for ALFY in cell migration. Wound healing analysis for 28 hours revealed that ALFY\textsuperscript{KO} cells showed reduced wound closure (Fig. 3A, B), but similar proliferation compared to HeLa\textsuperscript{WT} cells (Fig S3A), indicating that ALFY\textsuperscript{KO} cells have a diminished ability to migrate. By manually tracking individual cells, we found that ALFY\textsuperscript{KO} cells migrate faster than HeLa\textsuperscript{WT} cells, but lack directionality, and thereby fail to migrate into the wound (y-direction, FMI\textsubscript{y}) (Fig. 3C-D, Fig. S3B). This is reminiscent of the axon pathfinding defects observed in the Alfy KO mice, where axons failed to respond to chemotaxic cues (Dragich et al., 2016).

Moreover, recruitment of cortactin, an actin branching-promoting protein that is recruited to the leading edge of cells during cell migration (Ammer and Weed, 2008), was significantly abrogated in ALFY\textsuperscript{KO1-1} cells compared to HeLa\textsuperscript{WT} cells, but rescued by expression of EGFP-ALFY (Fig. 3E-F), indicating that ALFY promotes formation of a leading edge during directional cell migration, in line with its enrichment in cell protrusions at the leading edge (Fig. 1C) and reduced directionality of ALFY\textsuperscript{KO} cells (Fig. 3C-D, Fig. S3B).
ALFY regulates cell attachment and integrin glycosylation

As ALFY localizes to cellular protrusions and regulates cell migration, we asked if ALFY might regulate turnover of FAs, being important for cells to attach and detach during cell migration. Indeed, there was a significant reduction in the attachment of ALFYKO cells compared to HeLaWT cells (Fig. 4A). To determine if this is caused by changes in FA proteins, we examined the expression levels of integrin proteins in ALFYKO and rescue cells. Interestingly, migration of the protein bands of Integrin-α5, Integrin-αV and Integrin-β1 was reduced in the ALFYKO2 clones and this was rescued upon re-expression of EGFP-ALFY (Fig. 4B). Moreover, increased protein levels of Integrin-αV and Integrin-β3, as well as reduced levels of paxillin, were detected in the ALFYKO1 cell line (Fig. 4B). It is not clear why the two ALFYKO clones affect different integrins, but as ALFY is a very large protein with several transcripts, we cannot rule out that the two gRNAs (targeting different exons) result in expression of a part of ALFY that potentially could have dominant negative functions, although no truncated proteins were observed with the available antibodies (Fig. S1E-F). However, the changes in integrins observed upon ALFY-depletion could be rescued by expression of full-length ALFY, indicating that ALFY regulates trafficking and possibly post-translational modifications of integrins. To address the latter, WT and ALFYKO cells were treated with PNGaseF enzyme to cleave N-glycans or calf intestinal phosphatase (CIP) to remove phosphate. The mobility shift difference of Integrin-α5 and Integrin-β1 in ALFYKO cells was completely lost in cells treated with PNGaseF, while CIP treatment had no effect (Fig. 4C), demonstrating that ALFY regulates N-glycosylation of integrins. Both Integrin-α5 and Integrin-β1 contain several potential N-linked glycosylation sites, and their glycosylation can affect the heterodimerization and binding properties, cell migration and adhesion (Isaji et al., 2009, Isaji et al., 2006, Marsico et al., 2018, Hang et al., 2017). The mechanism of how ALFY regulates glycosylation of integrins is unclear, but its colocalization with Integrin-α5-mScarlet-I in cell protrusions and in intracellular structures (Fig. 4D), as well as with RAB5A and EEA1 (Fig 2A-B, Movie 1), suggest that it might regulate endocytosis of integrins from the plasma membrane and their transport to the recycling compartments. We observed close apposition and short-term colocalization of ALFY with the recycling endosome marker RAB11A and late endosome marker RAB7A (Fig. S1G-H, Movie 2-3), supporting this hypothesis. Glycosylation of integrins in RAB11 positive recycling endosomes (Kitano et al., 2021) and trans-Golgi network or perinuclear recycling compartments, where integrins transverse during long-loop recycling (De Franceschi et al., 2015), can explain the different glycosylation pattern of integrin-α5β1 in ALFYKO cells. Interestingly, N-
glycosylation of Integrin-α5 has been reported to regulate EGFR activation (Hang et al., 2015), which may explain the effect of ALFYKO on EGFR turnover (Fig. S2F-J).

Live-cell imaging revealed enrichment of ALFY in highly mobile structures in close proximity to paxillin-decorated FAs, suggesting they can deliver or remove components of FAs (Fig. S3C). It is established that migrating cells must remove their assembled adhesions at the rear of the cell and reintroduce them at the leading edge of the cell to migrate forward (Bretscher, 1989, Bretscher, 1992). As we observed EGFP-ALFY positive structures both in the leading and trailing edge of cells (Fig. 1C), we speculate that ALFY is involved in the bi-directional trafficking to and from early endosomes (Fig. 4E). Further experiments are however required to determine the nature of the ALFY-mediated trafficking and its role in glycosylation of integrins.

In this study, we report for the first time the intracellular localization and dynamics of full length ALFY in live cells. We show that ALFY localizes to cell protrusions and early endosomes, from where it seems to regulate proper sorting of integrins, thereby controlling directional cell migration and attachment (Fig. 4E). Our results thus provide an explanation for the defective neuronal migration and pathfinding phenotypes observed in mice lacking Alfy (Dragich et al., 2016).

Materials and methods

Cell lines, media and inhibitors

The HeLa T-Rex Flp-In cell line (Tighe et al., 2008) was obtained as a kind gift from A. Thige and S.S. Taylor, University of Manchester, UK and U2OS T-Rex Flp-In cell line (Malecki et al., MCB 2006) was a kind gift of Stephen C. Blacklow, Harvard Medical School, USA. The cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% foetal bovine serum (FBS), 5 U/ml penicillin and 50 μg/ml streptomycin, as well as 5 μg/ml blasticidin (Invitrogen) and 100 μg/ml zeocin (Invitrogen) to maintain the TET-repressor and the Flp-In site. The cell lines with a stably integrated gene in the Flp-In site, were kept in 5 μg/ml blasticidin and 100 μg/ml hygromycin B (VWR), and gene expression induced by a 17-24 hours treatment with 1 μg/ml doxycycline (Clonetech/AH) or tetracycline (Sigma, T7660). Puromycin (Sigma) was used at 2 μg/ml for selection of cells transfected with the CRISPR plasmid. VPS34IN1 (Selleckchem) was used at 5 ug/ml for 2 hours. Bafilomycin A1 (BafA1; Enzo, BML-CM110-0100) was used at 100 nM for inhibition of lysosomal degradation. All cell lines were routinely tested for contamination.
**Antibodies**

The following primary antibodies were used for immunofluorescence and/or immunoblotting: rabbit anti-ALFY (Novus Biologicals, NB1-03332, Bethyl Laboratories, A301-869A, Abcam, ab84888, LSBio, Cat. No LS-C483076 and Simonsen, 2004), anti-EEA1 (BD biosciences, 610457), anti-myc (Abcam, ab9132), anti-cortactin (millipore upstate, 05-180), anti-paxillin (abcam, ab32084), anti-β-actin (Cell signaling, #3700), anti-flag (Sigma, F1804), anti-GFP (Clontech, #632381), anti-integrin-α4 (Cell Signaling Technology, 8440T), anti-integrin-α5 (Cell Signaling Technology, 4705T), anti-integrin-αV (Cell Signaling Technology, 4711T), anti-integrin-β1 (Cell Signaling Technology, 9699T), anti-integrin-β3 (Cell Signaling Technology, 13166T), anti-integrin-β5 (Cell Signaling Technology, 3629T), anti-EGFR (Fitzgerald, 20-ES04), anti-transferrin receptor (Zymed, 13-6890, 1:5000), Anti-mouse (Starbright 700, BioRad, 12004158), anti-rabbit (DyLight 800, Thermo fisher Scientific, SA5-10044), anti-sheep (DyLight 594, Thermo fisher Scientific, SA510056), anti-rabbit (HRP, Jackson, 111-035-144), anti-mouse (Cy3, Jackson, 715-165-151), anti-rabbit (DyLight 649, Jackson, 711-495-152), anti-mouse (AlexaFluor647, Invitrogen, A31571), anti-goat (Cy5, Jackson, 705-175-147). Hoechst staining 33342 (Invitrogen, H1399).

**Plasmids and cloning**

ALFY was amplified by PCR from a human cDNA library in four fragments (base pair 1-3028, 2256-5425, 5123-7570 and 7355-10581), and cloned into the PCR blunt II TOPO vector (Invitrogen). For generation of LIR and FYVE domain mutants, mutagenesis was performed using QuickChange Lightning Multi Site-Directed mutagenesis kit (Agilent) on the constructs containing the target ALFY sequence. ALFY fragments, with or without LIR/FYVE mutations, were joined by Gibson assembly in pENTR1A (Invitrogen) to generate full length ALFY. The resulting full-length ALFY insert was verified by sequencing. ALFY constructs lacking PH-BEACH or WD40-FYVE domains were generated by restriction enzyme cloning from the full-length pENTR-ALFY. pDest-FlpIn-tdNGFlag-ALFY and pDest-FlpIn-EGFP-ALFY were generated using Gateway recombination cloning (Invitrogen) from pENTR1A-ALFY WT, LIR-/FYVE-mutants or ΔPH-BEACH/ΔWD40-FYVE mutants. Vectors for stable transfection of mScarlet-I-RAB5A, mScarlet-I-RAB7A, mScarlet-I-Rab11A, mScarlet-I-LC3B, mScarlet-I-GABARAP, mScarlet-I-SQSTM1 were generated by restriction enzyme subcloning of corresponding cDNA into pLVX lentiviral backbone. To generate the Gateway destination vector expressing tandem dimer NeonGreen (pDestFlpIn-tdNGFlag), the cDNA encoding two copies of NeonGreen with 3xFlag tag as a linker.
sequence was synthesized de novo (ThermoFisher GeneArt Gene Synthesis) and clone instead of
HA tag into pDestFlpInHA vector. pLVX-paxillin-mScarlet-I and pLVX-Integrin α5-mScarlet-I were
generated by Gibson assembly. All constructs were verified by Sanger sequencing. pCMV-
VSV-G, was a gift from Bob Weinberg (Addgene plasmid # 8454), psPAX2 was a gift from Didier
Trono (Addgene plasmid # 12260). pcDNA3-myc-RAB5 WT and -Q79L plasmids were a kind gift
from Professor Harald Stenmark at the Institute for Cancer Research, Oslo University Hospital,
Norway.

Virus production and transduction

10⁶ HEK-FT cells were plated in a 10cm dish and transfected next day with 1.6 µg of each of
pCMV-VSV-G, psPAX2 and transfer plasmids. Media was changed after 24 hours and lentivirus
containing media was collected and filtered through Acrodisc 0.45 µm Supor membrane syringe
filter at 48 and 72 hours after transfection. For cell infection 10⁵ HeLa or U2OS cells were plated in
wells of 6-well plate and next day cell media was exchanged for 2 ml fresh media with 0.5 ml of
lentivirus-containing media and 8 µg/ml of polybrene (SantaCruz Biotech). Media was changed 24
hours after infection for fresh media containing 2 µg/ml of puromycin. The pool of puromycin-
selected cells was used for experiments.

Generation of stably transfected cell lines

HeLa T-Rex FlpINWT, ALFYKO and U2OS cells were co-transfected with pOG44 Flp-recombinase
expression vector (Invitrogen) and pDEST-FlpIN-EGFP-ALFY, LIRmut, FYVEmut, ΔPH-BEACH
or ΔWD40-FYVE (for HeLa cells) or pDest-FlpIN-tdNGFlag-ALFY (for U2OS cells) in a ratio
10:1 using X-tremeGene9 DNA Transfection reagent (Roche, XTG9-RO). 24 hours post
transfection, the cells were split to four 10 cm dishes and medium containing 200 µg/ml
Hygromycin B (VWR) was added to select for cells containing the gene of interest stably integrated
into the genome. Individual clones were picked 2 weeks after selection and tested by western
blotting and confocal imaging for EGFP-ALFY expression. mScarlet-I-RAB5A, mScarlet-I-
RAB7A, mScarlet-I-RAB11A, mScarlet-I-LC3B, mScarlet-I-GABARAP, mScarlet-I-SQSTM1,
and Paxillin-mScarlet-I were generated by infection with lentiviral particles as described in the
Virus production and transduction section. Lipofectamine 2000 (Invitrogen, 11668019) was used
for transient transfections.
**Generation of ALFY KO cell line by CRISPR/Cas9**

To generate the ALFY KO cell line, CRISPR plasmids were made as described in the Zhang lab cloning protocol (Ran et al., 2013). Two independent single-guide RNAs (#1: 5’-GATCGGGAGCGTTTTAGAGG-3’, #2: 5’-GCAGAGTGAAGAAGCCAGTAG-3’) were designed using the crispr.mit.edu CRISPR design tool and obtained from Sigma (Fig. S1D). The guides were cloned into the hSpCas9-2A-Puro V2.0 (px459) plasmid (Addgene, 62988) and transfected into HeLa TRex FlpIN cells using X-tremeGene9 transfection reagent (Roche) according to the manufacture’s protocol. After 24 hours, cells were treated with selection medium containing 2.0 μg/ml puromycin. After 3 days, puromycin-resistant cells were seeded as single cell per well density in a 96-well plate by serial dilution. Several clones were tested, and the knock-out clones were confirmed by western blotting.

**Cell lysis and Western blotting**

For western blot analysis, cells grown to confluency were harvested in lysis buffer (150 mM NaCl, 1% Triton X-100, 1 mM EDTA and 50 mM Tris HCl pH 7.4) supplemented with complete protease inhibitor cocktail (Roche 05056489001) for 10 min on ice. The lysates were centrifuged at 15000 x g for 10 min at 4°C to pellet cell debris. Protein concentration of supernatant was measured using the BCA protein assay (Pierce, 23225) to ensure loading of equal amounts on SDS-PAGE. Following SDS-PAGE, western blotting was performed using primary antibodies of fluorophore- or HRP-conjugated secondary antibodies for detection and analysis with the Chemidoc™ MP Imaging System (BioRad). Prior to detection, membranes containing HRP-conjugated antibodies were incubated with Supersignal™ West Dura Extended duration substrate (ThermoFischer scientific, 34075) for 5 minutes.

For western blots of EGFR, cells were serum starved for 2 hours to induce expression of EGFR before treatment with 50 ng/ml EGF (Santa Cruz Biotechnology, sc-4552) for the indicated time points. The cells were subsequently lysed and prepared for western blotting as described above.

For treatment with PNGaseF and calf intestinal phosphatase (CIP) 3x10⁵ HeLa WT or ALFY KO²⁹ cells were plated in wells of 6 well plates and lysed in 100 µl/well of 50 mM TrisCl pH 7.5, 150 mM NaCl, 1% TritonX-100 the next day. Cell lysates were precleared by centrifugation at 12000g for 5 min. For treatment with PNGaseF, 9 µl of cell lysate was
mixed with 1 µl of glycoproteins denaturing buffer (NEB, B1704S), heated to 100°C for 10 minutes, then cooled down on ice, supplemented with 2 µl of 10x GlycoBuffer2 (NEB, B3704S), 2 µl of 10% NP40, 1 µl of PNGaseF (NEB, P0709S) and 5 µl of water and incubated for 1 hour at 37°C. For treatment with calf intestinal phosphatase, 9 µl of cell lysate was supplemented with 1.1 µl of 10x NEBuffer2 and 1 µl of calf intestinal phosphatase (NEB, M0290S) and incubated for 1 hour at 37°C.

**Immunoprecipitation**

For immunoprecipitation, cells were lysed in a buffer containing 150 mM NaCl, 1% Triton X-100 and 50 mM Tris HCl (pH 8.0) supplemented with complete protease inhibitor cocktail (Roche, 05056489001) (500 µl/10 cm dish). The lysates were incubated rotating at 4°C for 30 minutes. The lysates were centrifuged at max speed for 10 minutes at 4°C and the supernatants were incubated with 5 µl anti-ALFY antibodies rotating at 4°C overnight. Immunoprecipitation was performed by incubating 20 µl Protein G Dynabeads (Invitrogen, 10003D) to the lysates for 1 hour rotating at 4°C. The bound protein was collected using a DynaMag magnet (12321D) and washed three times in lysis buffer. The beads were washed twice with PBS and resuspended in 50 µl PBS before mass spectrometry analysis or western blot analysis.

**Transferrin (Tfn) recycling and flow cytometry analysis**

To measure Tfn recycling, WT and ALFY KO cells were first placed on ice for 10 minutes, before treatment with 10 µg/ml 555-Tfn (Invitrogen, T35352) for 15 minutes. The cells were then washed with PBS and chased for the indicated time periods in cell culture conditions at 37°C. After chase, the cells were trypsinized, fixed with 4 % PFA and centrifuged for 3 minutes at 500 x g to pellet the cells. The cells were washed in PBS and separated into single cells by passing them through a cell strainer cap attached to a 5 ml tube (falcon, 352235). The cells were analyzed by flow cytometry using the NovoCyte flow cytometer from Acea Biosciences.
Rhodamine-EGF degradation analysis

Cells grown in glass bottom 8-well chambers were treated with 50 ng/ml rhodamine-EGF (Invitrogen, E348) for 15 minutes at 37°C, followed by either 60, 30 or 0 minutes chase, before fixation with 4 % paraformaldehyde (PFA; Polysciences, 18814-10) for 15 minutes on ice. 1X PBS was added to each well and images of rhodamine-EGF were acquired using an Andor Dragonfly 505 spinning-disk confocal microscope using a NIKON Apo TIRF 60x/1.49 oil immersion objective. The whole cell volume was imaged by acquiring a series of z-stacks with 0.3 µm axial distance. For image analysis of rhodamine-EGF, Z-stack maximum intensity projections were used to segment Rhodamine-EGF vesicles in cells. The average count and size (area) of rhodamine-EGF-positive vesicles were quantified using CellProfiler (v. 4.1.3) (Carpenter et al., 2006).

Immunofluorescence, confocal microscopy and colocalization analysis

Cells were grown on glass cover slips or in glass bottom 8-well chambers and treated as described before fixation in 4 % paraformaldehyde (PFA; Polysciences, 18814-10) for 15 minutes at room temperature. The PFA was quenched in 0.05 M NH$_4$Cl for 10 minutes followed by permeabilization for 5 minutes in PBS with 0.05 % saponin. Immunostaining was performed by incubating the fixed cells with the indicated primary and corresponding secondary antibodies diluted in PBS with 0.05 % saponin. The cells were subsequently stained for 10 minutes with 1 g/ml Hoechst diluted in PBS. Cover slips were mounted in Prolong Diamond Antifade Mountant (Invitrogen, p36965). The cells were analyzed using a Zeiss LSM 710 confocal microscope with a 63X objective lens.

Colocalization histograms were generated using ImageJ (Fiji), by drawing a line through the structure of interest and obtaining the grey values (intensity) per pixel using the plot profile function. The intensities of the two overlapping channels were plotted against each other for generating the graphs.

Live cell imaging

Cells grown in 8-well Lab-Tek II chambered coverglass were imaged live in FluoreBrite DMEM medium (Gibco) on Andor Dragonfly 505 high speed confocal platform equipped with Okolab cell incubator with temperature, CO2 and humidity control, using NIKON Apo TIRF 60x/1.49 oil
immersion objective. The spinning disk confocal mode was used for all figures, except Fig. 1B, right panel, where TIRF mode with 100 nm penetration depth was used.

Wound healing assay and directional analysis

Cells were seeded in 96 well ImageLock™ plates (Essen Bioscience). When the cells reached a confluent monolayer, wounds were made using the WoundMaker tool (Essen Bioscience). To remove cell debris, the medium was changed, and the plate was subsequently placed in the IncuCyte® S3 Live Cell microscope (Sartorius) for imaging. One image per well was captured every 10 minutes for 28 hours to monitor cell migration into the wound. Relative wound density was quantified using the IncuCyte® Software S3 (Sartorius), measuring the spatial cell density in the wound area relative to the spatial cell density outside of the wound area at every time point. The data is presented as the mean relative wound density using the mean of three replicate wells from three independent experiments.

Manual tracking of cells from the wound healing assay was performed using the Image J software with the Manual Tracking plugin and the Chemotaxis and Migration tool (ibidi GmbH). Persistence of cells is calculated by dividing the Euclidean distance by the accumulated distance obtained by the ibidi Chemotaxis and Migration tool.

For directional analysis of cells using immunofluorescence, cells were grown to confluency in 2-well silicone inserts with 500 µm cell free gap (ibidi). The inserts were removed, and the cells were incubated in fresh medium for 4-5 hours, before fixation and staining against cortactin as mentioned above to mark the leading edge. Analysis by confocal imaging, 5-10 images were captured from three independent experiments. For quantification, cells with cortactin-positive leading edge were manually counted and compared to the total number of cells in each field of view, and a total of 60-100 cells from each condition were counted.

Cell proliferation assay

Cell proliferation was measured by MTT assay using a Cell proliferation and cytotoxicity kit (Boster, AR1156) according to the manufacturer’s instructions. Cells were seeded in triplicates at different cell densities in a 96-well plate. 24 hours after seeding, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to the medium and left for 4 hours for the viable, proliferating cells to form purple insoluble formazan crystals. The crystals were solubilized
overnight, and the concentration of the resulting colored solution was determined by measuring the optical density at 560 nm.

**Cell attachment assay**

The different cell lines were trypsinized and stained with CellMask™ Deep Red Plasma membrane Stain (Invitrogen, C10046) before subsequent seeding in a 96 well plate (6 replicates per cell line). The cells were incubated for 30 minutes to allow cells to attach to the well bottom before 3x washes in PBS to remove the non-attached cells. The 96 well plate was scanned using Odyssey CLx Imager using the 700 laser and total intensity per well was quantified using the Image Studio™ Software as a measure of the remaining cells.

**Statistical analysis**

Significance was determined using GraphPad Prism 8.0.1 and p-values were derived from two-tailed t-tests for unpaired samples and considered statistically significant at $p \leq 0.05$ (see figure legends for further details).

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Author contributions

KS, SP, CB, EMH, AKD and AHL designed and performed the experimental research. LRB analyzed imaging data. KS, SP and CB drafted the article and made the figures. AY provided reagents and revised the manuscript. AS designed the project, analyzed the data and contributed to writing the final version of the manuscript.

Disclosure of Potential Conflicts of Interest

The authors declare that they have no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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**Figure 1 ALFY localizes to cellular protrusions**

A. Domain structure of ALFY. ALFY contains a long N-terminus, followed by a conserved C-terminus with a PH-BEACH-domain, five WD repeats including an LC3-interacting region (LIR) and a PtdIns(3)P-binding FYVE-domain.

B. HeLa cells with inducible expression of EGFP-ALFY were treated with tetracycline for 24 hours and imaged live with spinning disc confocal (left) or TIRF (right) imaging modes. Arrowheads highlight accumulated fluorescent signal in cellular protrusions. Scale bar = 10 µm.

C. HeLa KO1-1 cells stably transfected with 3xFlag-EGFP-ALFY were imaged live for 100 minutes. Arrows illustrate the movement direction of leading and lagging ends of the cell. Scale bar = 10 µm.

D. Western blot of HeLa T-Rex FlpINWT and ALFYKO1-1 cell lines generated by CRISPR-Cas9. *indicates unspecific protein bands.

E. Western blot of HeLa T-Rex FlpINWT, ALFYKO1-1 and ALFYKO1-1 rescue cells treated or not with doxycycline to induce expression of EGFP-ALFY, LIRmut and FYVEmut. * indicates unspecific bands.

F. ALFYKO1-1 rescue cell lines were treated with doxycycline for 24 hours to induce expression of EGFP-ALFY, LIRmut, FYVEmut, ΔWD40-FYVE and ΔPH-BEACH followed by live cell imaging. EGFP-ALFYWT cells were treated or not with VPS34IN1 for 2 hours (left panel) prior to imaging. Scale bar = 10 µm.

G. Quantification of the % of cells with EGFP-ALFY in protrusions from the images in F. (mean ± SEM from 35-100 cells per cell line).
Figure 2 ALFY localizes to early endosomes in a PtdIns(3)P dependent manner

A. ALFY<sup>KO1-1</sup>-EGFP-ALFY rescue cells were fixed and immunostained against EEA1 and analyzed by confocal microscopy. Yellow arrowheads: EGFP-ALFY structures positive for EEA1. Colocalization histogram from the two vesicles marked with the white arrow. Scale bar = 10 µm.

B. ALFY<sup>KO1-1</sup>-EGFP-ALFY cells stably expressing mScarlet-I-RAB5 were treated with tetracycline overnight and analyzed with live cell imaging. mScarlet-I-RAB5 and EGFP-ALFY-positive puncta are indicated by arrowheads, and colocalization is shown in the histogram.

C. HeLa ALFY<sup>KO1-1</sup> cells rescued with EGFP-ALFY, EGFP-ALFY LIR<sup>mut</sup> or EGFP-ALFY FYVE<sup>mut</sup> were transiently transfected with myc-RAB5<sup>Q79L</sup>, fixed 18 hours post transfection, and immunostained against myc for visualization of the RAB5<sup>Q79L</sup> structures before confocal microscopy analysis. Scale bars = 10 µm. Colocalization histograms from the indicated myc-RAB5<sup>Q79L</sup> structures.

D. ALFY<sup>KO1-1</sup>-EGFP-ALFY cells with stable expression of mScarlet-I-tagged LC3B were treated with tetracycline and imaged live. Colocalization was measured from the indicated structures. Scale bar = 10 µm.

E. ALFY<sup>KO1-1</sup>-EGFP-ALFY cells with stable expression of mScarlet-I-tagged GABARAP were treated with tetracycline and imaged live. Colocalization was measured from the indicated structures. Scale bar = 10 µm.
Figure 3 ALFY is required for directional cell migration

A. Wound healing analysis of HeLa\textsuperscript{WT} and ALFY\textsuperscript{KO1-1} cells using Incucyte\textregistered live cell imaging system. The mean relative wound density was quantified from three replicates from three independent experiments. The timepoints marked by a dotted square in the left panel are shown as a magnified graph to the right (mean ± SEM, \( n = 3 \)). * \( p < 0.05 \) by multiple \( t \)-tests.

B. Representative images from (A.) showing the wound densities of HeLa\textsuperscript{WT} and ALFY\textsuperscript{KO1-1} at 0 and 28 hours.

C. Graphs representing movements of HeLa\textsuperscript{WT} and ALFY\textsuperscript{KO1-1} cells obtained by tracking individual cells in the images from (A.) using the Chemotaxis and migration tool (Ibidi).

D. Manual tracking of images from wound healing assays of HeLa\textsuperscript{WT}, ALFY\textsuperscript{KO1-1}, ALFY\textsuperscript{KO2-6}, ALFY\textsuperscript{KO2-9} and ALFY\textsuperscript{KO2-11}, showing velocity, directionality and forward migration index along the y-axis. (mean ± SEM, \( n = 3 \)). * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \) and **** \( p < 0.0001 \) by student’s \( t \)-test).

E. Analysis of leading edge formation in HeLa\textsuperscript{WT} cells, ALFY\textsuperscript{KO1-1} cells and ALFY\textsuperscript{KO1-1} rescue cells with doxycycline-induced expression of EGFP-ALFY, immunostained against cortactin for marking the leading edge. The white lines indicate the introduced wound.

F. Quantification of the % of cells from (E) with cortactin at the leading edge. (mean ± SEM, \( n = 3 \)). ** \( p < 0.01 \) and *** \( p < 0.001 \) by student’s \( t \)-test).
Figure 4 ALFY regulates cell attachment and integrin trafficking

A. Quantification of attached cells (stained with CellMask) (mean ± SEM, $n=4$. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ by student’s $t$-test).

B. Analysis of integrin protein levels from HeLa$^{WT}$ and ALFY$^{KO1}$, ALFY$^{KO2}$, ALFY$^{KO2-9}$ and ALFY$^{KO2-11}$ cells as well as ALFY$^{KO2-9}$-EGFP-ALFY rescue cells. The blots are representative of three independent experiments.

C. HeLa$^{WT}$ and ALFY$^{KO2-9}$ were treated or not with PNGaseF or CIP for 1 hour at 37°C, followed by Western blot analysis using the indicated antibodies.

D. Representative live cell image of ALFY$^{KO2}$-EGFP-ALFY rescue cells, co-expressed with Integrin-α5-mScarlet-I. Arrowheads indicate structures positive for both. Scale bar = 10 µm.

E. Model for role of ALFY in cell migration. ALFY localizes to cell protrusions in the leading and trailing edge of migrating cells and to early endosomes. By being present on these structures, ALFY regulates the proper sorting and bi-directional trafficking of adhesion proteins such as integrins, thereby controlling directional cell migration.
**Fig. S1.**  
A. HeLa cells stably transfected with EGFP-ALFY were treated with doxycycline for 24 hours to induce expression of EGFP-ALFY, followed by western blot analysis.  
B. Western blot analysis of different clones of U2OS cells stably expressing full length ALFY fused to a tag containing a tandem-dimer of NeonGreen with 3xFlag as a linker (tNGFlag).  
C. Representative image of U2OS cells stably expressing the tNGFlag-ALFY.  
D. Two CRISPR guides targeting ALFY were used to generate the ALFY KO cell lines. One guide targeting exon 6 (coding aa 102-138) (generating ALFYKO 2-6, ALFYKO 2-9 and ALFYKO 2-11) and one guide targeting exon 47 (coding aa 2481-2535) (generating ALFYKO 1-1).  
E. Western blot analysis of endogenous ALFY protein levels to confirm KO of the ALFYKO 2-6, ALFYKO 2-9 and ALFYKO 2-11 cell lines. To verify the correct ALFY band the cells were treated with either control siRNA or siRNA against ALFY.  
F. Immunoprecipitation of endogenous ALFY from HeLa T-Rex FlpInWT and ALFYKO 1-1 cells was performed using different antibodies recognizing either the N- or C-terminus of ALFY, followed by western blotting with the same antibodies.  
G. and H. ALFYKO1-1-EGFP-ALFY cells stably expressing mScarlet-I-Rab7A (G.) or mScarlet-I-Rab11A (H.) were treated with tetracycline overnight and analysed with live cell imaging.  
I. ALFYKO1-1-EGFP-ALFY cells, treated overnight with tetracycline and imaged live in DMEM, 37°C (left) or 10 min after exchange of media for cold PBS (right).
**Fig. S2.** A. ALFYKO1-1-EGFP-ALFY cells stably expressing mScarlet-I-SQSTM1 were treated with tetracycline overnight and analyzed with live cell imaging. Scale bar = 10 µm. B. Colocalization histograms of EGFP-ALFYWT and myc-RAB5Q79L, generated from the EGFP-ALFY vesicles in Fig. 2C marked with a small arrow. C. 555-transferrin recycling in WT and ALFYKO1-1 analyzed by flow cytometry (n=3 for T=0 and 15 min, n=2 for T=5, 10 and 20 min). D. Western blot analysis of transferrin receptor protein levels in WT and ALFYKO1-1 cells starved or not in the presence or absence of the lysosomal inhibitor BafA1. E. Quantification of the TIR protein bands in D (n=3, p<0.05 by student’s t-test). F. WT and ALFYKO1-1 cells were treated with Rhodamine-EGF and chased for the indicated time points before fixation and imaging. Graph shows number of Rh-EGF spots (mean values of n=3 independent experiments). G. Quantification of Rh-EGF area (mean values of n=3 independent experiments). H. Representative images of the cells treated with Rhodamine-EGF (quantified in F and G). I. WT and ALFYKO1-1 cells were treated with EGF for the indicated time points before western blot analysis of the EGFR protein levels. J. The EGFR protein bands from I. were quantified (n=3, p<0.05 by student’s t-test).
Fig. S3. A. HeLa WT and ALFY KO 1-1 cells were seeded at the indicated cell densities and cell proliferation was measured after 24 hours using the MTT-assay, measuring the absorbance of solubilized formazan crystals at 560 nm. (n=4, p<0.05 by student’s t-test). B. Graphs representing the movement of HeLa WT, ALFY KO 2-6, ALFY KO 2-9, and ALFY KO 2-11 cells obtained by tracking cells from three individual wound healing experiments, using the manual tracking plugin of ImageJ and the Chemotaxis and migration tool (Ibidi). C. ALFY KO 1-1 EGFP-ALFY cells stably expressing paxillin-mScarlet-I were treated with tetracycline overnight and analyzed with live cell imaging. Scale bar = 10 µm.
Movie 1. Live cell imaging of HeLa cells stably transfected with EGFP-ALFY and mScarlet-I-RAB5A
Movie 2. Live cell imaging of HeLa cells stably transfected with EGFP-ALFY and mScarlet-I-RAB7A
Movie 3. Live cell imaging of HeLa cells stably transfected with EGFP-ALFY and mScarlet-I-RAB11A