Syntaxins 3 and 4 mediate vesicular trafficking of α5β1 and α3β1 integrins and cancer cell migration

PAUL DAY, KRISTA A. RIGGS, NAZARUL HASAN, DEBORAH CORBIN, DAVID HUMPHREY and CHUAN HU

Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine, Louisville, KY 40202, USA

Received March 17, 2011; Accepted May 10, 2011

DOI: 10.3892/ijo.2011.1101

Abstract. Integrins, a family of heterodimeric receptors for cell adhesion to the extracellular matrix (ECM), play key roles in cell migration, cancer progression and metastasis. As transmembrane proteins, integrins are transported in vesicles and delivered to the cell surface by vesicular trafficking. The final step for integrin delivery, i.e., fusion of integrin-containing vesicles with the plasma membrane, is poorly understood at the molecular level. The SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins syntaxins 1, 2, 3 and 4 are present at the plasma membrane to drive vesicle fusion. In this study, we examined the roles of syntaxins 1, 2, 3 and 4 in vesicular trafficking of α5β1 and α3β1 integrins. We showed that syntaxins 2, 3 and 4 were expressed in HeLa cervical adenocarcinoma cells and PANC-1 pancreatic adenocarcinoma cells. In migrating HeLa and PANC-1 cells, syntaxins 2, 3 and 4 co-localized with the lipid raft constituent G_{M1} ganglioside at the leading edge. siRNA knockdown (KD) of syntaxins 3 and 4, but not of syntaxin 2, in HeLa cells reduced cell surface expression of α5β1 and α3β1 integrins and accumulated the integrins in cytoplasmic vesicles, indicating that syntaxins 3 and 4 mediate vesicular trafficking of α5β1 and α3β1 integrins to the cell surface. In addition, KD of syntaxins 3 and 4 inhibited cell adhesion to fibronectin, suppressed chemotactic cell migration and triggered apoptosis. Collectively, these data suggest that syntaxins 3- and 4-dependent integrin trafficking is important in cancer cell migration and survival, and may be a valuable target for cancer therapy.

Introduction

Metastasis, the spreading of cancer from a primary site to distant organs, is responsible for 90 percent of cancer deaths. Metastasis consists of a series of sequential steps, including local tumor growth, migration and invasion of cancer cells through the basement membrane and into surrounding tissues, intravasation into lymphatic and blood vessels, survival and spread in the circulation, and extravasation and establishment of secondary colonies at distant sites (1). Integrins are major receptors for cell adhesion to the extracellular matrix (ECM) proteins, such as fibronectin, laminin, collagen and vitronectin (2). Integrins are profoundly involved in the metastatic cascade, especially in cancer cell migration and invasion. During cell migration, integrins mediate cell adhesion to the ECM at the leading edge and serve as traction points to move the cell body forward (3,4). Although their cytoplasmic domains have no intrinsic kinase activity, integrins are important mediators of cell signaling in addition to anchoring the cell to ECM. The binding of ECM ligands to the extracellular domains of integrins leads to integrin clustering and activation (outside-in activation), which recruits and activates signaling molecules including the non-receptor tyrosine kinases focal adhesion kinase and Src (5-7).

Integrins are heterodimers of α and β subunits, and each heterodimer has its own ligand subset and function. After synthesis in the endoplasmic reticulum, integrins are transported in vesicles and delivered to the plasma membrane by vesicular trafficking. Like other receptors, integrins at the plasma membrane are constantly endocytosed, transported into endosomes, and then recycled back to the surface (8). Endocytic recycling of integrins is particularly important during cell migration when the net forward movement of the cell leads to an accumulation of integrins towards the cell rear. To provide traction at the cell front, integrins are randomly endocytosed along the plasma membrane, and recycled and exocytosed at the protruding front of the cell (9-15).

Integrin endocytosis at the plasma membrane can take place through clathrin-coated pits, caveolin or lipid rafts (8,16,17). Integrin recycling to the cell surface occurs by multiple mechanisms (8). In 'long-loop recycling', integrins such as α5β1 are delivered to early endosomes, then to the perinuclear recycling endosomes that contain the GTPase Rab11 (18-20), before being returned to the plasma membrane. With certain stimuli, integrins such as αvβ3 can be recycled directly from early endosomes to the plasma membrane to complete 'short-loop recycling' (21). The goal of our study is to identify the proteins that mediate the final step in integrin delivery, the fusion of integrin-containing vesicles with the plasma membrane. A large body of work has demonstrated that the interactions of SNARE (soluble N-ethylmaleimide-sensitive

Correspondence to: Dr Chuan Hu, Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine, 319 Abraham Flexner Way, Room 504, Louisville, KY 40202, USA
E-mail: chuan.hu@louisville.edu

Key words: integrin, cancer cell migration, vesicular trafficking, SNARE, syntaxin

factor attachment protein receptor) proteins on vesicles (v-SNAREs) and on target membranes (t-SNAREs) drive intracellular vesicle fusion (22-24). The cytoplasmic domains of v- and t-SNAREs form extremely stable complexes to promote membrane fusion (25-27). Individual members of the SNARE family localize to distinct subcellular organelles (28), suggesting that each SNARE has a selective role in vesicular trafficking events.

Several SNARE proteins have been implicated in integrin trafficking. Expression of the catalytic light chain of tetanus toxin that selectively cleaves the v-SNARE VAMP3, impairs cell migration and reduces cell surface α5β1 integrin in fibroblast cells (29,30). We have shown that small interfering RNA (siRNA)-induced silencing of VAMP3 inhibits pancreatic cancer cell migration and β1 integrin trafficking (31). These findings indicate that VAMP3 is involved in integrin trafficking. In addition, we reported that the v-SNARE VAMP2 mediates α5β1 trafficking in HeLa cells and VAMP2-dependent integrin trafficking is critical in cell migration (32). However, it remains unclear which t-SNARE proteins mediate the fusion of integrin-containing vesicles with the plasma membrane.

Four members of the syntaxin t-SNARE family, i.e., syntaxins 1, 2, 3 and 4, are localized at the plasma membrane for vesicle fusion (28). Syntaxin 1 drives synaptic exocytosis (33), while syntaxin 2 has been implicated in dense core granule exocytosis in platelets (34). Syntaxin 3 is involved in apical membrane trafficking in polarized epithelial cells (35,36), and syntaxin 4 mediates insulin-stimulated translocation of the glucose transporter GLUT4 in adipocytes (37). A recent study showed that in macrophages t-SNAREs syntaxin 4 and SNAP-23 partner with VAMP3 to deliver α5β1 integrin to the cell surface (38).

Using cancer cells as models, in the current study we examined the function of syntaxins 1, 2, 3 and 4 in integrin trafficking. We show that both syntaxins 3 and 4 play active roles in α5β1 and α3β1 integrin trafficking and in cancer cell migration.

Materials and methods

Cell culture and transfection. HeLa and PANC-1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). HeLa cells were cultured in Minimum Essential Medium α (MEMα) with 10% fetal bovine serum (FBS). PANC-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/l glucose and 10% FBS. The anti-β1 integrin mouse hybridoma cell line P5D2, developed by Dr Wayner, was obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa.

The day before siRNA transfection, HeLa cells were seeded in 6-well plates at a density of $2.5x10^5$ cells/well or 24-well plates at a density of $5x10^4$ cells/well. siRNAs were transfected at 10 nM using Lipofectamine RNAiMAX according to the manufacturer's instructions (Invitrogen). The AllStars Negative Control siRNA, and the predesigned siRNA oligos syntaxin 2 siRNA (Hs_STX2_1, targeting sequence TTCGAGCCAAGTTAAAGGCTA) and syntaxin 3 siRNA (Hs_STX3_1, targeting sequence AAGGGCCAACAACGTC CGGAA) were purchased from Qiagen. A 21-nucleotide siRNA oligo that targets the human syntaxin 4 sequence GGAAT AAAGTCCAGGAGTT was synthesized by Dharmacon Thermo Scientific.

Immunocytochemistry. For syntaxin and lipid raft staining, HeLa and PANC-1 cells were seeded on sterile 12-mm glass coverslips contained in 24-well plates. Twenty-four hours after seeding, cell monolayers were wounded with a 1000 μl pipet tip and returned to culture. Twenty-four hours later, the cells were fixed with 4% paraformaldehyde in PBS^{++} (PBS supplemented with 0.1 g/l $CaCl_2$ and 0.1 g/l $MgCl_2$) for 10 min at room temperature. A 0.2% Triton X-100 solution in PBS^{++} was used to permeabilize the cells. Cells were then blocked in 10% FBS in PBS^{++} for 30 min. Lipid rafts were stained using FITC-conjugated cholera toxin B (Sigma-Aldrich Co.) at a dilution of 1:400. Rabbit polyclonal antibodies (pAbs) to syntaxins 2, 3 and 4 (Synaptic Systems GmbH) were used at a dilution of 1:100. Rhodamine (TRITC)-conjugated anti-rabbit antibodies (Jackson Immunoresearch Laboratories) were used at a dilution of 1:500. Confocal images were obtained at x60 on an Olympus laser scanning confocal microscope.

For integrin staining, HeLa cells were seeded on sterile 12-mm glass coverslips contained in 24-well plates. Seventy-two hours after siRNA transfection, the cells were fixed and permeabilized. α5 integrin was stained using the monoclonal antibody (mAb) MAB1999 (Millipore) at a 1:100 dilution, and α3 integrin was stained using the mAb MAB2056 (Millipore) at a 1:100 dilution. FITC-conjugated anti-mouse secondary antibodies were used at a dilution of 1:500.

Immunoblotting. After 72 h of siRNA transfection, HeLa cells were lysed in 2X SDS-PAGE buffer. Whole cell lysates of 40 μl were separated by SDS-PAGE. For experiments shown in Figs. 1A and 2C, whole cell lysates were prepared by incubating cells for 30 min in lysis buffer (50 mM Tris-pH 8.0, 150 mM NaCl and 1% NP-40) containing the complete protease inhibitor cocktail (Roche). Protein concentrations were determined by the Bio-Rad DC Protein assay. Lysates of 30 μg were loaded into each lane of the SDS-PAGE gel. After electrophoresis, resolved proteins were transferred to nitrocellulose membranes and blocked with 5% milk in TBS. The membranes were blotted with a rabbit pAb to syntaxin 2, a rabbit pAb to syntaxin 3, a mouse mAb to syntaxin 4 (Sigma-Aldrich Co.), a rabbit pAb to α5 integrin (Santa Cruz Biotechnology), a mouse mAb to β-actin (Sigma-Aldrich Co.), and HRP-conjugated secondary antibodies. Membranes were incubated with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific Pierce) and exposed to Kodak Biomax Light film.

Flow cytometry. The levels of cell surface integrins were measured using flow cytometry as previously described (39). HeLa cells were seeded in 24-well plates. Seventy-two hours after siRNA transfection, the cells were fixed with 1% paraformaldehyde in PBS for 15 min, and then blocked in 10% FBS in PBS for 15 min. The cells were incubated with 20 μg/ml of control mouse IgG (Sigma), anti-α3 integrin mAb, anti-α5 integrin mAb, or anti-β1 integrin mAb P5D2 for 60 min at room temperature. After three washes with 0.05 % heat-inactivated BSA in PBS, the cells were labeled with FITC-conjugated secondary antibodies (1:200 dilution) for

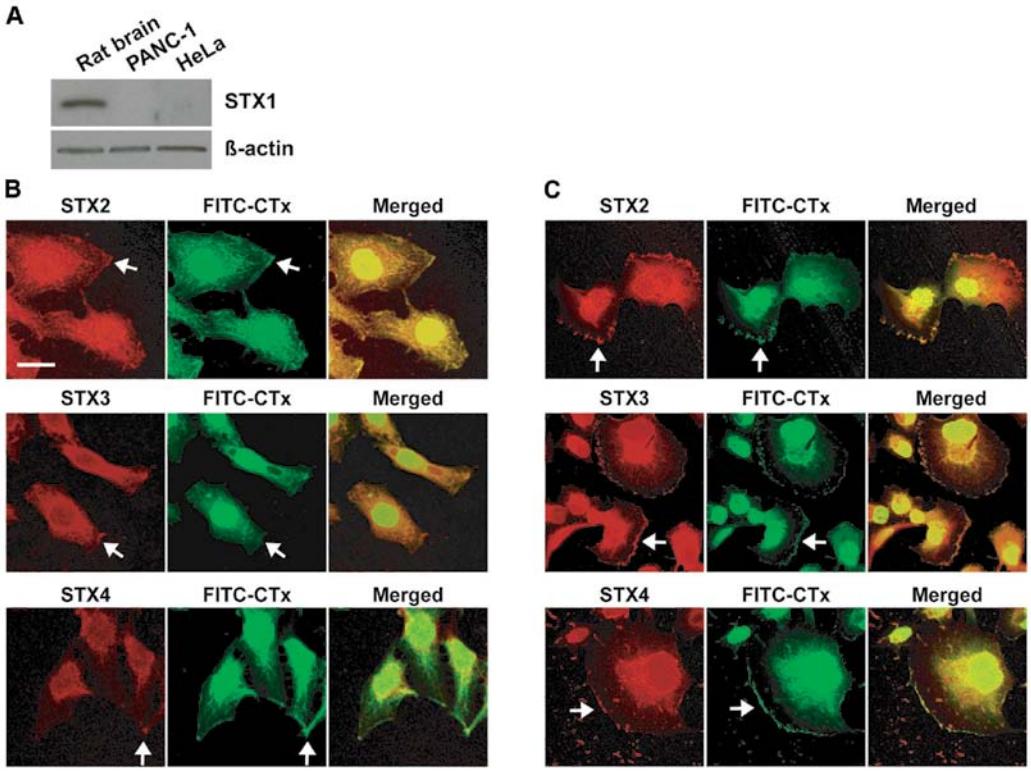

Figure 1. Colocalization of syntaxins (STX) 2, 3 and 4 with lipid rafts. (A) Syntaxin 1 is not expressed in PANC-1 and HeLa cells. Whole lysates of 30 μg of rat brain, PANC-1 cells and HeLa cells were immunoblotted with an antibody to syntaxin 1. The same membrane was reprobed with an antibody to β-actin as a loading control. (B and C) Syntaxins colocalize with the G_{M1} ganglioside in lamellipodia. (B) HeLa and (C) PANC-1 cell monolayers were wounded with a pipet tip to stimulate migration. Twenty hours after wounding, the cells were permeabilized and dual stained with the FITC-conjugated cholera toxin B-subunit (FITC-CTx) and an antibody to syntaxins 2, 3 or 4. Representative confocal images are shown. Scale bar, 20 μm.

45 min. After three washes with 0.05% heat-inactivated BSA in PBS, the cells were scraped off the plates with a cell scraper. Ten thousand cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences) in the James Graham Brown Cancer Center. The mean fluorescence intensity of each sample was obtained using the CellQuest Pro software.

Cell proliferation assay. The day before siRNA transfection, HeLa cells were seeded in 96-well plates at a density of $2x10^3$ cells/well. 0, 1, 2, 3 and 6 days after transfection, cell proliferation was measured using the CellTiter 96 AQ_{ueous} One Solution Reagent (Promega). Briefly, the cell culture medium was replaced with the MEMα medium containing no phenol red. The CellTiter 96 AQ_{ueous} One Solution Reagent was added, and the cells incubated at 37˚C for 90 min. Absorbance of the medium at 490 nm was determined in a 96-well ELISA plate reader. Absorbance from wells containing only the MEMα medium but no cells served as the negative (blank) reading.

Cell adhesion assay. Each well of the 24-well plates was coated with 20 μg of fibronectin (BD Biosciences) for 1 h at 37˚C. The plates were rinsed with PBS and blocked with 2% heat-inactivated BSA for 1 h at 37˚C. Seventy-two hours after siRNA transfection, HeLa cells were harvested with trypsin/EDTA, added to the wells at a density of $1x10^5$ cells/well and allowed to adhere for various time points at 37˚C. Nonadherent cells were removed by gentle washing and the number of attached cells was measured using the CellTiter 96 AQ_{ueous} One Solution Reagent as described above.

Transwell migration assay. The transwell migration assay was performed as previously described (31) with minor modifications. MEMα serum-free medium containing 20 μg/ml of growth factor-reduced Matrigel (BD Biosciences) was added to the lower chambers of the 12-well format transwells (8 μm-pore, BD Biosciences) as chemoattractant. Forty-eight hours after siRNA transfection, HeLa cells were harvested with trypsin/EDTA, and added to the upper chambers at a density of $8x10^4$ cells/well. After 20 h at 37˚C, the transwells were fixed in methanol and stained with Giemsa stain solution. Cotton swabs were used to remove unmigrated cells from the top of the membranes. Ten random images were taken at x10 magnification on a light microscope to quantify the number of migrated cells for each transwell. The number of migrated cells per image was counted using the ImageJ software.

Apoptosis assay. Apoptotic cells were stained using the CaspACE™ FITC-VAD-FMK In Situ Marker according to the manufacturer's instructions (Promega). HeLa cells were seeded on sterile 12-mm glass coverslips contained in 24-well plates. Three days after siRNA transfection, the cells were incubated with CaspACE™ FITC-VAD-FMK at 10 μM for 20 min, and then fixed. Fluorescent images were collected on an Olympus laser scanning confocal microscope.

Results

Syntaxins 2, 3 and 4 colocalize with the lipid raft constituent G_{M1} ganglioside in lamellipodia. Among the four syntaxins

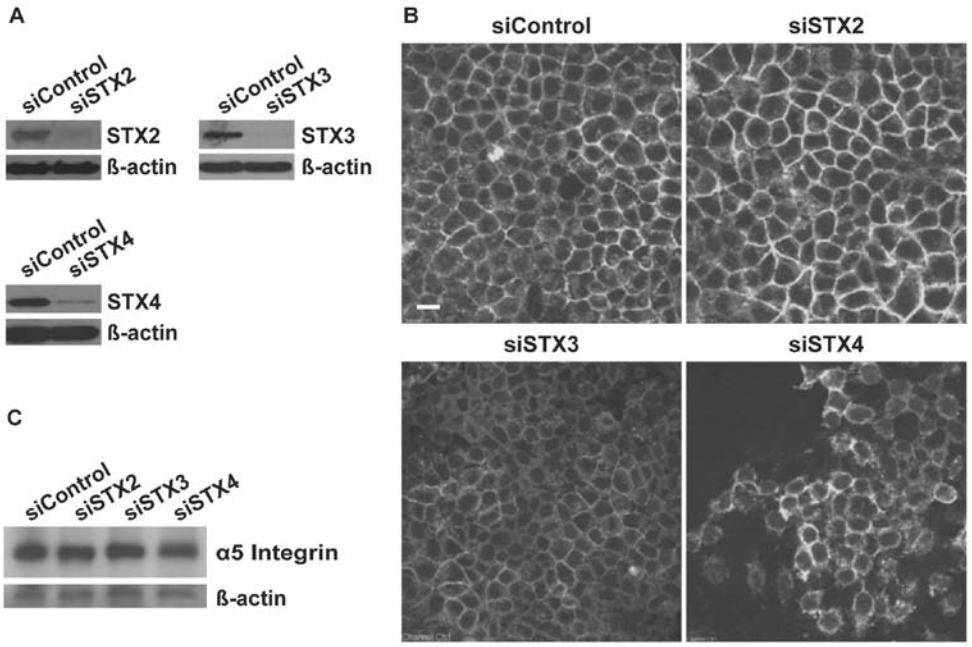

Figure 2. siRNA knockdown of syntaxins (STX) 3 and 4 diminishes cell surface expression of α5 integrin. (A) HeLa cells were transfected with a non-targeting control siRNA or a siRNA to syntaxins 2, 3 or 4. Seventy-two hours after transfection, whole cell lysates of the cells were immunoblotted with an antibody to syntaxins 2, 3 or 4. The same membranes were reprobed with an antibody to β-actin as a loading control. (B) The transfected cells were stained with an antibody to α5 integrin without permeabilization. Representative confocal images of 3 independent experiments are shown. In each experiment, images were collected at the same pinhole, detector gain and laser intensity. Scale bar, 50 μm. (C) 30 μg of whole cell lysates of the transfected cells were immunoblotted with an antibody to α5 integrin.

localized at the plasma membrane, syntaxin 1 is nervous tissue-specific. Indeed, immunoblotting analysis showed that syntaxin 1 is expressed abundantly in rat brain but not in HeLa or PANC-1 cells (Fig. 1A). Immunocytochemistry was used to determine the subcellular distribution of syntaxins 2, 3 and 4 in migrating cells. To stimulate migration, HeLa and PANC-1 cell monolayers were wounded. HeLa cells, and more prominently PANC-1 cells, formed lamellipodia when moving into the wounded areas (Fig. 1B and C). Syntaxins 2, 3 and 4 were detected in the plasma membrane as well as in cytoplasmic membrane-bound compartments. Interestingly, all three syntaxins were enriched in lamellipodia - the leading edge of migrating cells (arrows, Fig. 1B and C).

Lipid rafts are cholesterol- and glycosphingolipid-enriched membrane domains that have been implicated in the regulation of signal transduction and vesicular trafficking. Lipid rafts concentrate at the leading edge to establish front-rear polarity in migrating cells (40,41). When HeLa and PANC-1 cells were double stained with the syntaxin antibodies and FITC-conjugated cholera toxin B-subunit (FITC-CTx), which detects G_{M1} ganglioside highly enriched in lipid rafts (40), we found that syntaxins 2, 3 and 4 colocalized extensively with G_{M1} ganglioside in lamellipodia (Fig. 1B and C).

Syntaxins 3 and 4 knockdown (KD) inhibits α5β1 and α3β1 integrin trafficking. In order to determine the roles of syntaxins 2, 3 and 4 in integrin trafficking, we depleted the syntaxin proteins in HeLa cells with siRNAs. Western blot analysis showed that siRNA treatment depleted syntaxins 2, 3 and 4 proteins effectively (Fig. 2A). We then determined if syntaxins 2, 3 and 4 KD had any effects on the cell surface expression of α5 integrin. When syntaxin 2 was depleted, there was no significant difference in α5 integrin distribution at the plasma membrane compared to the control (Fig. 2B). Syntaxin 3 KD disrupted the ring-like distribution of α5 integrin, and reduced the intensity of α5 integrin staining. When syntaxin 4 was depleted, α5 integrin cell surface staining was also reduced and the ring of α5 integrin was dispersed (Fig. 2B). Compared with control cells, the levels of total cellular α5 were not altered in syntaxins 2, 3 or 4 KD cells (Fig. 2C). The reduction of the cell surface α5 but not of the total cellular α5 suggested that KD of syntaxins 3 and 4 disrupted the trafficking of α5 integrin to the plasma membrane.

If syntaxins 3 and 4 mediate the fusion of integrin-containing vesicles with the plasma membrane, KD of the syntaxins is expected to increase the localization of integrins in cytoplasmic vesicles. To analyze cytoplasmic integrin distribution, HeLa cells transfected with the control or syntaxin siRNAs were permeabilized, and stained with antibodies to α5 and α3 integrins. In control cells, small number of vesicles containing α5 and α3 integrins were detected (Fig. 3). Syntaxin 2 KD did not alter the quantity of α5 and α3-containing vesicles or integrin staining intensity of the vesicles. Strikingly, the number of α5 and α3-containing vesicles and the staining intensity of α5 and α3 integrins in the vesicles were increased dramatically in syntaxin 3 KD cells (arrows, Fig. 3). In syntaxin 4 KD cells, in addition to increased vesicular accumulation of α5 and α3 integrins (arrows, Fig. 3), disorganization of cytoplasmic membrane bound compartments was observed (arrowheads, Fig. 3). Since α5 and α3 integrins only assemble with the β1 subunit to form integrin receptors (2), the subcellular distribution and expression levels of α5 and α3 represent those of α5β1 and α3β1 integrins.

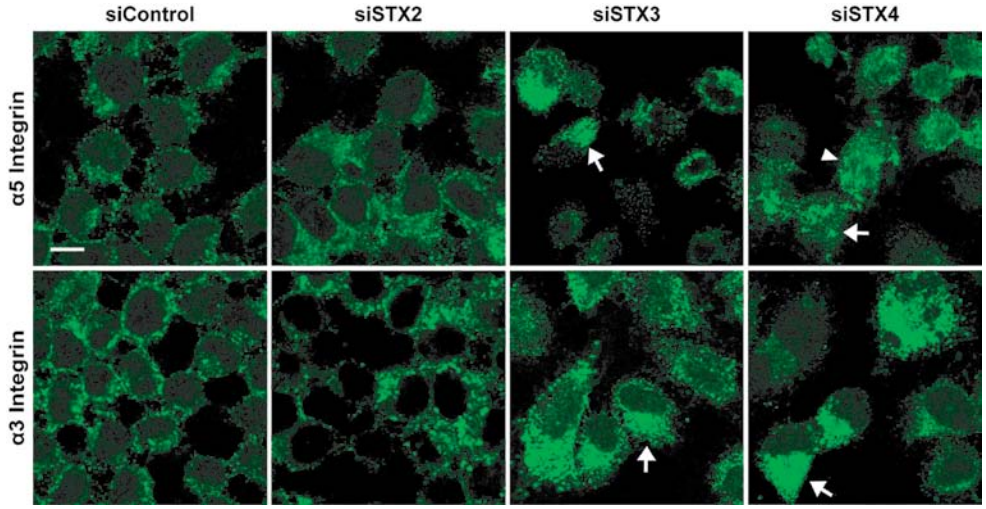

Figure 3. Accumulation of α5 and α3 integrins in cytoplasmic vesicles in syntaxins 3 and 4 knockdown cells. Seventy-two hours after transfection with the control, syntaxins 2, 3 or 4 siRNAs, HeLa cells were permeabilized and stained with an antibody to α5 integrin (top row) or α3 integrin (bottom row). Representative confocal images are shown. Scale bar, 20 μm.

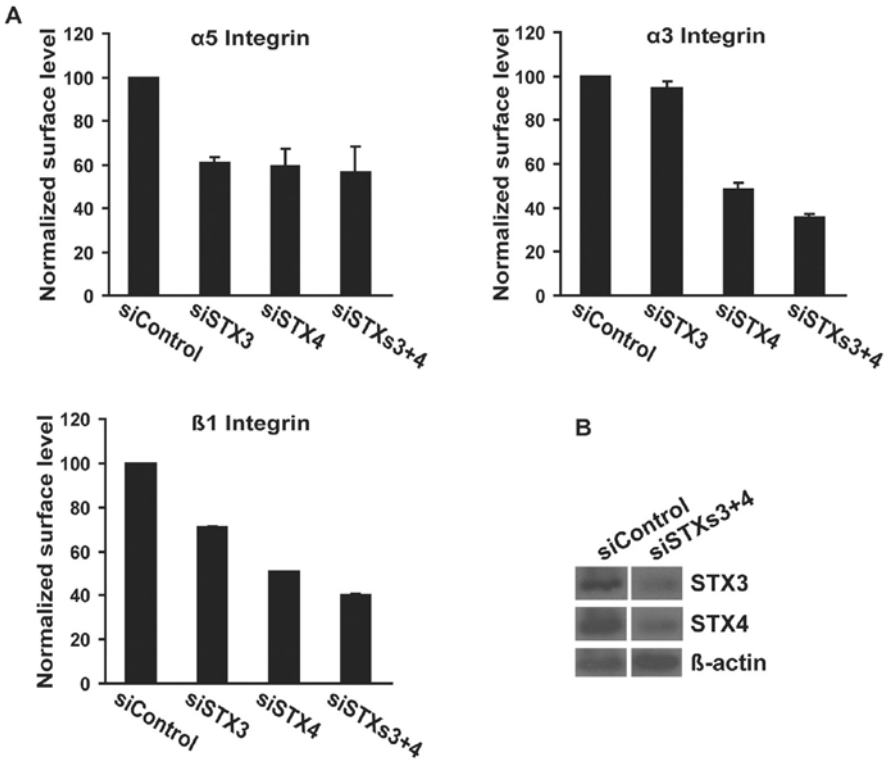

Figure 4. FACS analysis of cell surface integrins. (A) Seventy-two hours after transfection with the control, syntaxins (STX) 2, 3 or 4 siRNAs, or both syntaxins 3 and 4 siRNA, unpermeabilized HeLa cells were stained with non-specific control mouse IgG (blank control) or a monoclonal antibody to α3, α5 or β1 integrins, and then analyzed by flow cytometry. The mean fluorescence intensity of cell surface α3, α5 and β1 integrins in cells transfected with the syntaxin siRNAs was normalized to the intensity in cells transfected with the control siRNA. Error bars represent standard deviation of 2 independent experiments. (B) Seventy-two hours after transfection with both syntaxins 3 and 4 siRNAs, whole cell lysates of HeLa cells were immunoblotted with an antibody to syntaxins 3 or 4. The same membrane was probed with an antibody to β-actin as loading control. Shown are cropped lanes from the same immunoblotting images.

To quantitatively measure the effects of syntaxins 3 and 4 KD on cell surface integrin expression, we performed flow cytometric analysis (32). In accordance with the microscopic observations (Figs. 2 and 3), compared with control cells syntaxin 3 and 4 KD cells had a 39 and 41% reduction of α5 integrin cell surface staining, respectively (Fig. 4A). In addition, KD of syntaxins 3 and 4 reduced β1 integrin cell surface staining by 29 and 49%, respectively. While syntaxin 4 KD reduced α3 integrin cell surface staining by 51%, syntaxin 3 KD reduced α3 staining by 5% (Fig. 4A). Taken together, the reduction of cell surface α5β1 and α3β1 integrin expression (Figs. 2 and 4) and accumulation of the integrins in

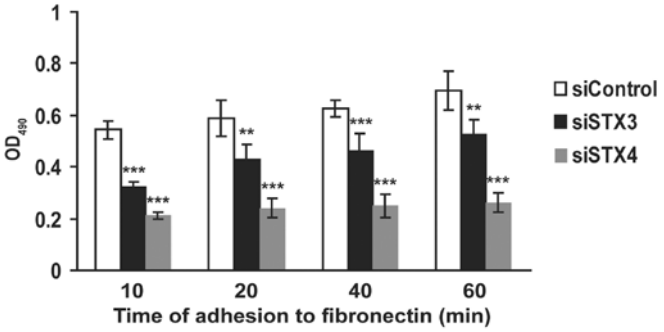

Figure 5. Knockdown of syntaxins (STX) 3 and 4 inhibits cell adhesion to fibronectin. Seventy-two hours after transfection with the control (white columns), syntaxin 3 (black columns) or syntaxin 4 (gray columns) siRNAs, HeLa cells were harvested and added to fibronectin-coated plates. At 10, 20, 40 and 60 min, unattached cells were washed away and the number of adherent cells was measured using a colorimetric assay as described in Materials and methods by absorbance at 490 nm. Error bars represent standard deviation of 3 independent experiments. $^{**}P<0.01$ vs. cells transfected with the control siRNA; $^{***}P<0.001$ vs. cells transfected with the control siRNA.

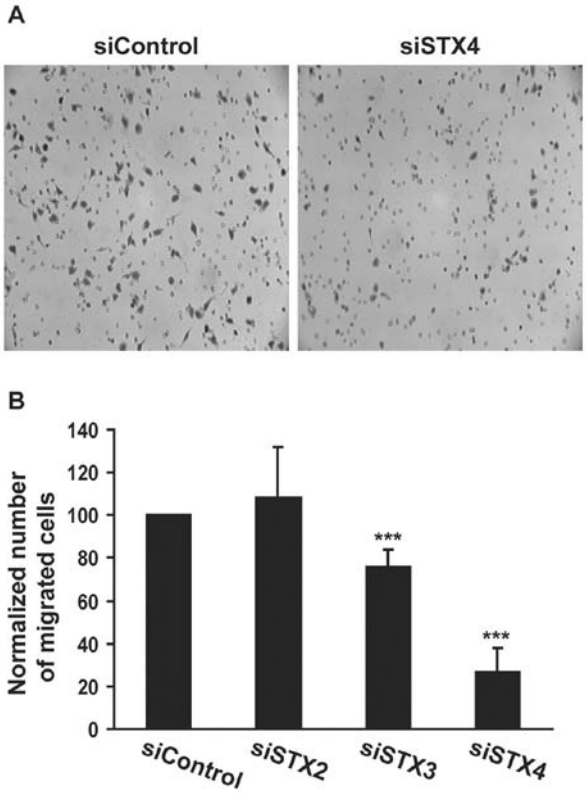

Figure 6. Knockdown of syntaxins (STX) 3 and 4 inhibits chemotactic cell migration. Forty-eight hours after transfection with the control, syntaxins 2, 3 or 4 siRNAs, HeLa cells were harvested and loaded to the top chambers of transwells. Matrigel (20 μg/ml) was included in the bottom chambers as chemoattractant. After 20 h, unmigrated cells were removed and migrated cells were Giemsa stained. (A) Representative images of migrated cells. (B) Random images were taken for each transwell, and the number of migrated cells transfected with syntaxins 2, 3 and 4 siRNAs was normalized to the number of migrated cells transfected with the control siRNA. Error bars represent standard deviation of 3 independent experiments. $^{***}P<0.001$ vs. cells transfected with the control siRNA.

cytoplasmic vesicles (Fig. 3) in syntaxins 3 and 4 KD cells indicated that syntaxins 3 and 4 mediate the trafficking of α5β1 and α3β1 integrins to the cell surface.

Because integrins traffic to the cell surface through multiple pathways (8), we sought to determine if syntaxins 3 and 4 play redundant roles in integrin trafficking. We reason that if syntaxins 3 and 4 function in distinct integrin trafficking pathways, KD of both syntaxins 3 and 4 would have additive effects on integrin trafficking compared with syntaxins 3 or 4 KD. On the other hand, if syntaxins 3 and 4 function in the same pathway, double KD is expected to have the same effects as single KD. To distinguish these mechanisms, we depleted syntaxins 3 and 4 simultaneously (Fig. 4B) and measured the double KD effects on cell surface α5, α3 and β1 integrin expression. Compared with single KD, double KD of syntaxins 3 and 4 had no additive effect on α5 cell surface expression (Fig. 4A), suggesting that syntaxins 3 and 4 function in the same pathway of α5β1 trafficking. Interestingly, the double KD reduced cell surface expression of α3 and β1 integrins significantly more than KD of syntaxins 3 or 4 (Fig. 4A), suggesting that syntaxins 3 and 4 function in different pathways of α3β1 trafficking.

Knockdown of syntaxins 3 and 4 inhibits cell adhesion and chemotactic migration. Integrins enable the cell to adhere to the ECM. α5β1 integrin is a receptor for fibronectin (42). Because KD of syntaxins 3 and 4 disrupted α5β1 trafficking, we next investigated if KD of syntaxins 3 and 4 affects cell adhesion to fibronectin. Syntaxin 3 KD impaired the cells' ability to adhere to fibronectin (a 41% decrease at 10 min) (Fig. 5), and syntaxin 4 KD showed an even larger effect (a 61% decrease at 10 min) (Fig. 5). These results provide functional evidence that syntaxins 3 and 4 mediate α5β1 trafficking.

In cell migration, integrins are delivered to the cell front by vesicular transport and polarized exocytosis (8). Using the transwell migration assay, we measured the effects of syntaxins 3 and 4 KD on chemotactic cell migration to Matrigel, which is mainly made of collagen IV and laminin. Syntaxin 3 and 4 KD inhibited chemotactic migration by 24 and 74%, respectively, whereas syntaxin 2 KD had no significant effect (Fig. 6B), suggesting that syntaxins 3 and 4-dependent integrin trafficking is important in chemotactic migration.

Knockdown of syntaxins 3 and 4 inhibits cell proliferation and triggers apoptosis. In addition to adhesion and migration, integrins regulate cell proliferation and survival (43). We performed cell proliferation assays to assess the effects of syntaxin 3 and 4 KD on proliferation. At 3 days after siRNA transfection, syntaxins 3 and 4 KD diminished HeLa proliferation by 16 and 45%, respectively. Six days after transfection, inhibition of proliferation increased to 36 and 86%, respectively (Fig. 7A). To determine if the reduction in cell number was due to apoptosis, HeLa cells transfected with the control, syntaxins 3 or 4 siRNAs were incubated with a FITC-conjugated caspase inhibitor VAD-FMK to label apoptotic cells (arrows, Fig. 7B). A small fraction of the control cells underwent apoptosis. Treatment with syntaxin 3 siRNA clearly increased the number of apoptotic cells. Furthermore, syntaxin 4 KD resulted in a large increase of apoptosis (Fig. 7B). These results show that KD of syntaxins 3 and 4 triggered apoptosis.

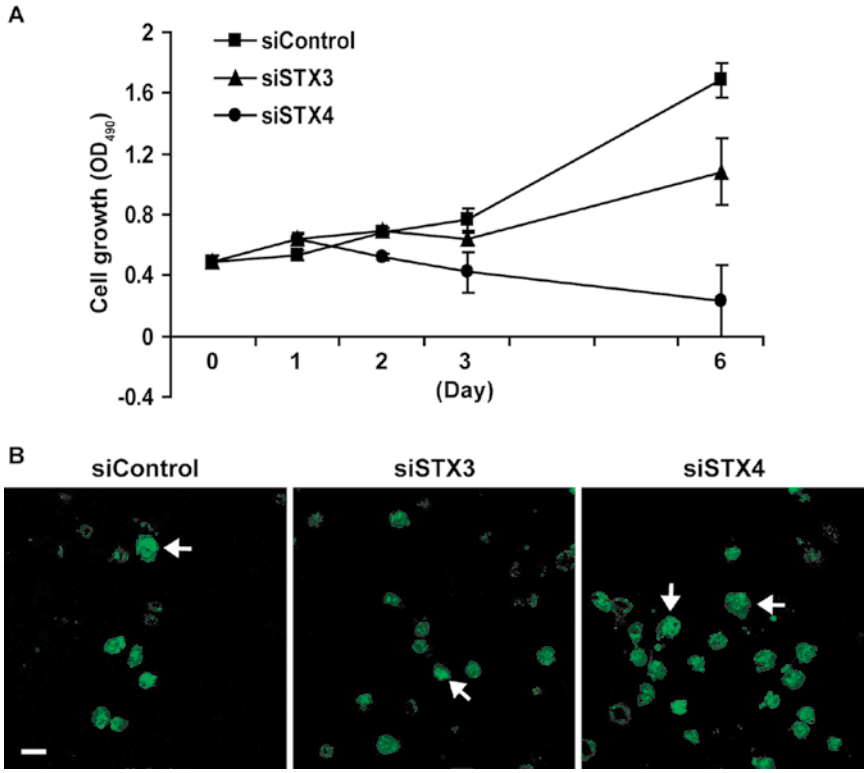

Figure 7. Proliferative and apoptotic responses of HeLa cells by syntaxins (STX) 3 and 4 knockdown. (A) 0, 1, 2, 3 and 6 days after transfection with the control, syntaxins 3 or 4 siRNAs, the number of living HeLa cells was determined as described in Materials and methods by absorbance at 490 nm. Error bars represent standard deviation of 3 independent replicates. (B) Three days after siRNA transfection, the CaspACE™ FITC-VAD-FMK In Situ Marker was added to label apoptotic cells. Representative confocal images are shown. Bar, 20 μm.

Discussion

In the current study, we examined the roles of syntaxins 2, 3 and 4 in vesicular trafficking of α5β1 and α3β1 integrins. In HeLa cervical adenocarcinoma cells and PANC-1 pancreatic adenocarcinoma cells, syntaxins 2, 3 and 4 colocalize with the lipid raft constituent G_{M1} ganglioside in lamellipodia. Depletion of syntaxins 3 and 4, but not syntaxin 2, reduced cell surface expression of α5β1 and α3β1 integrins in HeLa cells and accumulated the integrins in cytoplasmic vesicles, indicating that syntaxins 3 and 4 are both involved in α5β1 and α3β1 trafficking to the cell surface. In addition, depletion of syntaxins 3 and 4 inhibited cell adhesion to fibronectin and chemotactic cell migration, and triggered apoptosis, suggesting that syntaxins 3 and 4-mediated integrin trafficking is important in cancer cell migration and survival.

In migrating cells, lipid rafts are concentrated at the leading edge to establish front-rear polarity (40,41). Both syntaxins 3 and 4 have been shown to associate with lipid rafts (35,44). In mast cells, syntaxin 4 is equally distributed between raft and non-raft fractions, whereas syntaxin 3 is selectively enriched in rafts (45). We show here that syntaxins 2, 3 and 4 are enriched in lamellipodia at the leading edge where they colocalize with G_{M1} ganglioside. Since integrins and other membrane components are exocytosed actively at the leading edge (46), enrichment of the syntaxin t-SNAREs may facilitate polarized exocytosis.

In macrophages, syntaxin 4 forms a complex with VAMP3 and SNAP-23 to deliver α5β1 integrin to the cell surface (38). Our data confirm the role of syntaxin 4 in α5β1 trafficking in HeLa cells. Furthermore, we show that syntaxin 4 is involved in α3β1 trafficking, suggesting that syntaxin 4 mediates the trafficking of multiple integrins. For the first time, we report that syntaxin 3 takes part in α5β1 and α3β1 trafficking. To analyze if syntaxins 3 and 4 function in the same or independent pathways of integrin trafficking, we measured the effects of syntaxins 3 and 4 double KD on cell surface integrin expression. Double KD had the same inhibitory effect on α5β1 surface expression as syntaxins 3 or 4 KD, suggesting that syntaxins 3 and 4 function in the same α5β1 trafficking pathway. However, double KD reduced α3β1 surface expression significantly more than syntaxins 3 or 4 KD, suggesting that syntaxins 3 and 4 play a role in different pathways of α3β1 trafficking.

Although KD of syntaxins 3 and 4 had similar inhibitory effects on the cell surface expression of α5β1, syntaxin 4 KD had a larger inhibitory effect on the cell surface expression of α3β1, cell adhesion to fibronectin, chemotactic migration and cell proliferation than KD of syntaxin 3. Such differences are unlikely caused by more efficient depletion of syntaxin 4 protein, as immunoblotting analyses showed that both syntaxins 3 and 4 proteins were depleted effectively (Fig. 2A). These differences suggest that for the trafficking of certain types of integrins (e.g., α3β1) and in the chemotactic migration and cell survival, syntaxin 4 may have a more important role than syntaxin 3. Furthermore, the differential effects of syntaxins 3 and 4 KD on the cell surface expression of α5β1 and α3β1 indicate that these two integrins utilize different vesicular trafficking mechanisms.

v- and t-SNAREs form ternary complexes to drive vesicle fusion with the plasma membrane (22-24). Previous studies show that v-SNAREs VAMP2 and VAMP3 are involved in integrin trafficking (29-32). A recent study (38) and our current study show that t-SNAREs syntaxins 3, 4 and SNAP-23 take part in integrin trafficking. It is tempting to speculate that VAMP2, VAMP3, syntaxins 3, 4 and SNAP-23 form the protein machinery that mediates the fusion of integrin-containing vesicles with the plasma membrane. It is known that VAMP2 forms complexes with syntaxin 4 and SNAP-23 (47), and VAMP3 forms complexes with SNAP-23 and syntaxins 3 or 4 (48,49). Future studies are warranted to distinguish the specific role of each v-/t-SNARE complex in integrin trafficking.

Metastasis is responsible for 90% of cancer mortality. Metastatic cancer cells express an abnormal repertoire of integrins that favor survival, migration and invasion (43). For example, metastatic prostate cancer cells have elevated expression of α5β1 integrin (50) and α2bβ3 integrin that mediates binding to platelets (51). Because a major target of prostate cancer metastasis is the bone, prostate cancer cells express α2β1 and αvβ3, which interact with the bone ECM proteins collagen I and vitronectin, respectively (50,52). Because of the importance of integrins in cancer progression and metastasis (43,53), various anti-integrin agents, including monoclonal antibodies and small-molecule inhibitors such as cilengitide, are in clinical development for cancer treatment (54,55) and have so far shown mixed results (56). Our studies show that depletion of the SNARE proteins VAMP2, VAMP3, syntaxins 3 and 4 inhibits integrin trafficking and chemotactic migration of cervical and pancreatic cancer cells. We propose that SNARE-mediated integrin trafficking is a valuable target for cancer therapy.

Acknowledgements

This study was supported by a startup fund from the University of Louisville School of Medicine and CA135123 from the National Institutes of Health of USA (to C.H.).

References

1. Huber MA, Kraut N and Beug H: Molecular requirements for epithelial-mesenchymal transition during tumor progression. Curr Opin Cell Biology 17: 548-558, 2005.
2. Hynes RO: Integrins: bidirectional, allosteric signaling machines. Cell 110: 673-687, 2002.
3. Ridley AJ, Schwartz MA, Burridge K, *et al*: Cell migration: integrating signals from front to back. Science 302: 1704-1709, 2003.
4. Webb DJ, Parsons JT and Horwitz AF: Adhesion assembly, disassembly and turnover in migrating cells - over and over and over again. Nat Cell Biol 4: E97-E100, 2002.
5. Wozniak MA, Modzelewska K, Kwong L and Keely PJ: Focal adhesion regulation of cell behavior. Biochim Biophys Acta 1692: 103-119, 2004.
6. Zhao J and Guan JL: Signal transduction by focal adhesion kinase in cancer. Cancer Metastasis Rev 28: 35-49, 2009.
7. Mitra SK and Schlaepfer DD: Integrin-regulated FAK-Src signaling in normal and cancer cells. Curr Opin Cell Biol 18: 516-523, 2006.
8. Caswell PT and Norman JC: Integrin trafficking and the control of cell migration. Traffic 7: 14-21, 2006.
9. Regen CM and Horwitz AF: Dynamics of beta 1 integrin-mediated adhesive contacts in motile fibroblasts. J Cell Biol 119: 1347-1359, 1992.
10. Palecek SP, Schmidt CE, Lauffenburger DA and Horwitz AF: Integrin dynamics on the tail region of migrating fibroblasts. J Cell Sci 109: 941-952, 1996.
11. Lawson MA and Maxfield FR: Ca(2+)- and calcineurin-dependent recycling of an integrin to the front of migrating neutrophils. Nature 377: 75-79, 1995.
12. Pierini LM, Lawson MA, Eddy RJ, Hendey B and Maxfield FR: Oriented endocytic recycling of alpha5beta1 in motile neutrophils. Blood 95: 2471-2480, 2000.
13. Laukaitis CM, Webb DJ, Donais K and Horwitz AF: Differential dynamics of alpha 5 integrin, paxillin, and alpha-actinin during formation and disassembly of adhesions in migrating cells. J Cell Biol 153: 1427-1440, 2001.
14. Kiosses WB, Shattil SJ, Pampori N and Schwartz MA: Rac recruits high-affinity integrin alphavbeta3 to lamellipodia in endothelial cell migration. Nat Cell Biol 3: 316-320, 2001.
15. Woods AJ, White DP, Caswell PT and Norman JC: PKD1/PKCmu promotes alphavbeta3 integrin recycling and delivery to nascent focal adhesions. EMBO J 23: 2531-2543, 2004.
16. De Deyne PG, O'Neill A, Resneck WG, Dmytrenko GM, Pumplin DW and Bloch RJ: The vitronectin receptor associates with clathrin-coated membrane domains via the cytoplasmic domain of its beta5 subunit. J Cell Sci 111: 2729-2740, 1998.
17. Wary KK, Mariotti A, Zurzolo C and Giancotti FG: A requirement for caveolin-1 and associated kinase Fyn in integrin signaling and anchorage-dependent cell growth. Cell 94: 625-634, 1998.
18. Roberts MS, Woods AJ, Dale TC, Van Der Sluijs P, and Norman JC: Protein kinase B/Akt acts via glycogen synthase kinase 3 to regulate recycling of alpha v beta 3 and alpha 5 beta 1 integrins. Mol Cell Biol 24: 1505-1515, 2004.
19. Powelka AM, Sun J, Li J, *et al*: Stimulation-dependent recycling of integrin beta1 regulated by ARF6 and Rab11. Traffic 5: 20-36, 2004.
20. Yoon SO, Shin S and Mercurio AM: Hypoxia stimulates carcinoma invasion by stabilizing microtubules and promoting the Rab11 trafficking of the alpha6beta4 integrin. Cancer Res 65: 2761-2769, 2005.
21. Roberts M, Barry S, Woods A, van der Sluijs P and Norman J: PDGF-regulated rab4-dependent recycling of alphavbeta3 integrin from early endosomes is necessary for cell adhesion and spreading. Curr Biol 11: 1392-1402, 2001.
22. Rothman JE: Mechanisms of intracellular protein transport. Nature 372: 55-63, 1994.
23. Jahn R, Lang T and Sudhof TC: Membrane fusion. Cell 112: 519-533, 2003.
24. Bonifacino JS and Glick BS: The mechanisms of vesicle budding and fusion. Cell 116: 153-166, 2004.
25. Weber T, Zemelman BV, McNew JA, *et al*: SNAREpins: minimal machinery for membrane fusion. Cell 92: 759-772, 1998.
26. McNew JA, Weber T, Parlati F, *et al*: Close is not enough: SNARE-dependent membrane fusion requires an active mechanism that transduces force to membrane anchors. J Cell Biol 150: 105-117, 2000.
27. Hu C, Ahmed M, Melia TJ, Sollner TH, Mayer T and Rothman JE: Fusion of cells by flipped SNAREs. Science 300: 1745-1749, 2003.
28. Chen YA and Scheller RH: SNARE-mediated membrane fusion. Nat Rev Mol Cell Biol 2: 98-106, 2001.
29. Proux-Gillardeaux V, Gavard J, Irinopoulou T, Mege RM and Galli T: Tetanus neurotoxin-mediated cleavage of cellubrevin impairs epithelial cell migration and integrin-dependent cell adhesion. Proc Natl Acad Sci USA 102: 6362-6367, 2005.
30. Skalski M and Coppolino MG: SNARE-mediated trafficking of alpha5beta1 integrin is required for spreading in CHO cells. Biochem Biophys Res Commun 335: 1199-1210, 2005.
31. Luftman K, Hasan N, Day P, Hardee D and Hu C: Silencing of VAMP3 inhibits cell migration and integrin-mediated adhesion. Biochem Biophys Res Commun 380: 65-70, 2009.
32. Hasan N and Hu C: Vesicle-associated membrane protein 2 mediates trafficking of alpha5beta1 integrin to the plasma membrane. Exp Cell Res 316: 12-23, 2010.
33. Bennett MK, Garcia-Arraras JE, Elferink LA, *et al*: The syntaxin family of vesicular transport receptors. Cell 74: 863-873, 1993.
34. Chen D, Bernstein AM, Lemons PP and Whiteheart SW: Molecular mechanisms of platelet exocytosis: role of SNAP-23 and syntaxin 2 in dense core granule release. Blood 95: 921-929, 2000.
35. Lafont F, Verkade P, Galli T, Wimmer C, Louvard D and Simons K: Raft association of SNAP receptors acting in apical trafficking in Madin-Darby canine kidney cells. Proc Natl Acad Sci USA 96: 3734-3738, 1999.
36. Sharma N, Low SH, Misra S, Pallavi B and Weimbs T: Apical targeting of syntaxin 3 is essential for epithelial cell polarity. J Cell Biol 173: 937-948, 2006.

37. Olson AL, Knight JB and Pessin JE: Syntaxin 4, VAMP2, and/or VAMP3/cellubrevin are functional target membrane and vesicle SNAP receptors for insulin-stimulated GLUT4 translocation in adipocytes. Mol Cell Biol 17: 2425-2435, 1997.
38. Veale KJ, Offenhauser C, Whittaker SP, Estrella RP and Murray RZ: Recycling endosome membrane incorporation into the leading edge regulates lamellipodia formation and macrophage migration. Traffic 11: 1370-1379, 2010.
39. Hasan N, Corbin D and Hu C: Fusogenic pairings of vesicle-associated membrane proteins (VAMPs) and plasma membrane t-SNAREs - VAMP5 as the exception. PLoS One 5: e14238, 2010.
40. Manes S, Mira E, Gomez-Mouton C, *et al*: Membrane raft microdomains mediate front-rear polarity in migrating cells. EMBO J 18: 6211-6220, 1999.
41. Gomez-Mouton C, Lacalle RA, Mira E, *et al*: Dynamic redistribution of raft domains as an organizing platform for signaling during cell chemotaxis. J Cell Biol 164: 759-768, 2004.
42. Takada Y, Huang C and Hemler ME: Fibronectin receptor structures in the VLA family of heterodimers. Nature 326: 607-609, 1987.
43. Guo W and Giancotti FG: Integrin signalling during tumour progression. Nat Rev Mol Cell Biol 5: 816-826, 2004.
44. Chamberlain LH and Gould GW: The vesicle- and target-SNARE proteins that mediate Glut4 vesicle fusion are localized in detergent-insoluble lipid rafts present on distinct intracellular membranes. J Biol Chem 277: 49750-49754, 2002.
45. Pombo I, Rivera J and Blank U: Munc18-2/syntaxin3 complexes are spatially separated from syntaxin3-containing SNARE complexes. FEBS Lett 550: 144-148, 2003.
46. Bretscher MS: Moving membrane up to the front of migrating cells. Cell 85: 465-467, 1996.
47. Foster LJ, Yeung B, Mohtashami M, Ross K, Trimble WS and Klip A: Binary interactions of the SNARE proteins syntaxin-4, SNAP23, and VAMP-2 and their regulation by phosphorylation. Biochemistry 37: 11089-11096, 1998.
48. Galli T, Zahraoui A, Vaidyanathan VV, *et al*: A novel tetanus neurotoxin-insensitive vesicle-associated membrane protein in SNARE complexes of the apical plasma membrane of epithelial cells. Mol Biol Cell 9: 1437-1448, 1998.
49. Murray RZ, Kay JG, Sangermani DG and Stow JL: A role for the phagosome in cytokine secretion. Science 310: 1492-1495, 2005.
50. Knudsen BS and Miranti CK: The impact of cell adhesion changes on proliferation and survival during prostate cancer development and progression. J Cell Biochem 99: 345-361, 2006.
51. Trikha M, Raso E, Cai Y, *et al*: Role of alphaII(b)beta3 integrin in prostate cancer metastasis. Prostate 35: 185-192, 1998.
52. Zheng DQ, Woodard AS, Fornaro M, Tallini G and Languino LR: Prostatic carcinoma cell migration via alpha(v)beta3 integrin is modulated by a focal adhesion kinase pathway. Cancer Res 59: 1655-1664, 1999.
53. Hood JD and Cheresh DA: Role of integrins in cell invasion and migration. Nat Rev Cancer 2: 91-100, 2002.
54. Ruegg C and Mariotti A: Vascular integrins: pleiotropic adhesion and signaling molecules in vascular homeostasis and angiogenesis. Cell Mol Life Sci 60: 1135-1157, 2003.
55. Stupp R, and Ruegg C: Integrin inhibitors reaching the clinic. J Clin Oncol 25: 1637-1638, 2007.
56. Reynolds AR, Hart IR, Watson AR, *et al*: Stimulation of tumor growth and angiogenesis by low concentrations of RGD-mimetic integrin inhibitors. Nat Med 15: 392-400, 2009.