

REVIEW



Annexin A6—A multifunctional scaffold in cell motility

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ABSTRACT

Annexin A6 (AnxA6) belongs to a highly conserved protein family characterized by their calcium (Ca^{2+})-dependent binding to phospholipids. Over the years, immunohistochemistry, subcellular fractionations, and live cell microscopy established that AnxA6 is predominantly found at the plasma membrane and endosomal compartments. In these locations, AnxA6 acts as a multifunctional scaffold protein, recruiting signaling proteins, modulating cholesterol and membrane transport and influencing actin dynamics. These activities enable AnxA6 to contribute to the formation of multifactorial protein complexes and membrane domains relevant in signal transduction, cholesterol homeostasis and endo-/exocytic membrane transport. Hence, AnxA6 has been implicated in many biological processes, including cell proliferation, survival, differentiation, inflammation, but also membrane repair and viral infection. More recently, we and others identified roles for AnxA6 in cancer cell migration and invasion. This review will discuss how the multiple scaffold functions may enable AnxA6 to modulate migratory cell behavior in health and disease.

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Introduction

Cell migration is fundamental for the organization and maintenance of multicellular organisms. During embryonic development, wound healing or immune responses that protect against pathogens, cells need to be equipped with multifaceted approaches to respond to biochemical signals from their local environment in order to migrate through tissues or across epithelial cell layers. On the other hand, deregulated and aggressive migratory behavior of cancer cells is a critical feature driving metastatic events.^{1–4}

For cells to undergo migration, a very complex and coordinated set of protein-protein interactions, signaling and membrane trafficking events is required, which has been described in detail in excellent reviews by others.^{1–4} In brief, the dynamics of cellular movement are initiated by the formation of focal adhesions at the leading edge. This facilitates attachment to the extracellular matrix (ECM) and links the cellular actin cytoskeleton to the ECM. At the leading edge, localized cytoskeletal rearrangements then generate traction forces that allow gradual forward movement. Consequently, for cells to continuously move forward, coordinated assembly and disassembly of adhesions at the cell's front and rear is critical. For cellular motion, heterodimeric integrin

receptors composed of α and β subunits are central to focal adhesion structure and function, and their interactions with the ECM trigger both mechanical and chemical signals permitting cells to adhere and migrate. Hence, the transient nature and rapid turnover of focal adhesions requires integrins to be constantly internalized and recycled for redistribution at the leading edge.^{1–4} This complex interplay of focal adhesion dynamics, integrin trafficking and cytoskeletal re-organisations is organized by a plethora of regulatory membrane transport proteins including Rabs, ADP ribosylation factors (ARFs) and soluble NSF attachment protein (SNAP) receptor (SNARE) proteins, and is coupled to structural elements that link to the actin cytoskeleton, such as vinculin, talin and α -actinin. Moreover, membrane trafficking and cytoskeletal re-arrangements have to be coordinated with the recruitment and spatiotemporal signaling of focal adhesion kinase (FAK), Src, Rho-family GTPases, growth factor receptors, and their numerous regulators and effectors such as protein kinase C α (PKC α) and p120 GTPase activating protein (p120GAP).^{1–4}

Thus, in these specialized local areas at the leading edge, forward movement requires precise coordination in space and time to enable targeted delivery and

endocytosis of vesicles, regulation of actin dynamics and signal complex formation. To facilitate dynamic assembly of specialized membrane domains, proteins that can temporarily stabilize protein–protein or protein–lipid interactions in specific cellular sites are essential. Scaffold, adaptor and targeting proteins fulfil this critical function, as their capacity to simultaneously interact with proteins and lipids is ideal to recruit signaling proteins and their regulators/effectors to specific membrane microdomains. These multiple features provide the platform for targeted vesicle transport from and to the plasma membrane, as well as localized actin re-arrangements, altogether enabling forward movement in a spatiotemporal manner. Over the years, an array of scaffold proteins relevant for cell migration, including paxillin, RACKs, IQGAPs, liprins and caveolin, have been identified.^{5–8} This implicates that a diverse repertoire of scaffolds orchestrates coordinated dynamics during focal adhesion assembly. Moreover, in disease settings like cancer and wound healing, differential expression patterns of scaffold proteins are now also considered to be conducive or detrimental for the establishment of microenvironments that promote migratory behavior.^{5–8}

Annexins – a conserved family of proteins with diverse scaffolding functions

Annexins represent another protein family with various scaffolding functions important for cell motility. Like many other scaffolds, the modular domain organization of annexins supports protein–protein and protein–lipid interactions.^{8–11} Each annexin contains a distinctive leader sequence at the N-terminus, followed by four, eight for AnxA6, conserved repeats that build the C-terminal annexin core, containing Ca^{2+} and phospholipid binding sites. Upon Ca^{2+} elevation, these features permit their rapid translocation from the cytosol to the plasma membrane or intracellular membranes.^{8–11} In fact, the transient nature of Ca^{2+} spikes inside cells facilitates reversible membrane binding behavior of annexins, making them ideal to create short-lived platforms and protein complexes for the continuous and localized assembly and disassembly of specialized structures required for forward movement.

Most annexins are widely expressed, often at high levels, in multiple locations and with various interaction partners. Despite these intricate characteristics, annexins are now recognized to alter the localization and function of signaling proteins, to create specific membrane microenvironments, and trigger cytoskeletal re-arrangements.^{8–13} For more than 20 years, several annexins have been known to influence the adhesive and migratory behavior of cells. For instance, early studies implicated

AnxA1 to control the adhesion and migration of leucocytes, such as neutrophils and granulocytes, during the inflammatory response induced by glucocorticoids.^{14,15} Soon thereafter, AnxA2 was reported to affect the migratory behavior of endothelial cells and neoangiogenesis.^{16,17} Similarly, recombinant AnxA5 was initially shown to inhibit lung carcinoma cell migration and T cell adhesion.^{18,19} Strikingly, all these annexins contribute to cell adhesion and migration through different mechanisms. This includes prominent extracellular functions that do not require Ca^{2+} -dependent membrane association. In fact, in vivo studies from AnxA1, A2 and A5-deficient mouse models strongly support these extracellular activities to significantly affect adhesive and migratory cell behavior, which has been reviewed in detail elsewhere.^{20–23}

For each of the abovementioned annexins (AnxA1, A2, A5), abundant and rapidly expanding literature related to cell adhesion and migration exists, while little is known about the possible roles for AnxA6 in this context. However, recent findings from our laboratory and others provide exciting insights how AnxA6 may influence the ability of cells to migrate and adhere. In the following we will summarize and discuss the multiple functions that enable AnxA6 to modulate cell migration and adhesion, with possible implications for metastatic events in cancer.

Extracellular roles of AnxA6 in cell migration

AnxA6 represents the largest member of the annexin family and is highly expressed in most tissues.^{8–11,24,25} AnxA6 is predominantly located at the plasma membrane and the endosomal compartment, where it interacts with multiple proteins and lipids to organize membrane domains, signaling complexes, transient membrane-actin interactions as well as intracellular cholesterol homeostasis.^{8,9,24–28} All of these interactions and regulatory functions appear relevant for cell proliferation, survival, differentiation, inflammation, membrane repair and viral infection, which has been reviewed in detail.^{8–11,23–25}

More recently, we and others identified some of these intracellular AnxA6 functions to contribute significantly to the migratory and adhesive behavior of fibroblasts and several cancer cell lines^{29,30} Yet, the first reports of AnxA6 affecting cell adhesion and migration came from studies implicating extracellular AnxA6 activities. When characterizing the molecular properties of metastatic cells, AnxA6 and other annexins were identified on the cell surface of metastatic RAW117 lymphoma cells.³¹ Anti-AnxA6 antibodies partially blocked Ca^{2+} -dependent adhesion of these cells to endothelial layers,

indicating that AnxA6 was a cell surface component that could possibly function as an adhesion molecule on migrating cancer cells.³¹ Soon thereafter, purified AnxA6 as well as other annexins were reported to bind glycosaminoglycans, such as chondroitin sulfate, heparin and heparin sulfate,^{32,33} which are ECM based molecules believed to contribute to adhesion of migrating cells. Moreover, ectopic expression of AnxA6 in A431 cells, an epithelial adenocarcinoma cell line which lacks endogenous AnxA6,^{26,34} conferred the ability to attach to chondroitin sulfate chains.³³ Flow cytometry analysis indicated that a certain pool of AnxA6 proteins could indeed be exposed on the external cell surface of A431 cells.³³ More recently, AnxA6 knockdown in triple-negative BT-549 breast cancer cells, lacking estrogen (ER) and progesterone receptors as well as ErbB2, reduced cell adhesion to fetuin-A, a prominent factor in serum considered to promote cell attachment of tumor cells.³⁵ The interaction of cells with immobilized fetuin-A seems to be mediated by cell surface annexins, including AnxA6 and AnxA2.³⁵⁻³⁷ In follow-up studies, Sakwe and colleagues examined the migratory behavior of AnxA6-depleted BT-549 cells.³⁰ AnxA6 depletion in these cells was associated with increased cell proliferation, which was in line with tumor suppressor activities of AnxA6 in epidermal growth factor receptor (EGFR) overexpressing A431, ER-negative breast cancer cells (BCC), head and neck squamous cell carcinoma lines (HNSCC), but also commonly used HeLa and HEK293 cell lines with moderate EGFR levels.^{28,38} In addition, AnxA6-depleted BT-549 showed normal adhesion to fibronectin (FN) and laminin, but cell spreading on fetuin-A coated surfaces was strongly inhibited. The latter correlated with reduced motility and invasiveness in vitro, altered vinculin staining and FAK signaling, indicating compromised focal adhesion assembly on fetuin-A coated surfaces.³⁰ Altogether these studies suggest that a pool of extracellular AnxA6 protein may bind to several proteoglycans or other proteins like fetuin-A to support the adhesive and migratory properties of cancer cells (Table 1).

It is yet unclear if these interactions of AnxA6 on outer cell surface membranes are relevant for in vivo cell attachment, where enrichment of ECM proteins like FN, laminin, and collagen most likely favor integrin-mediated adhesion. Annexins are generally considered intracellular proteins and do not contain signal sequences that facilitate secretion. However, substantial amounts of some annexins, including AnxA1, AnxA2 and AnxA5, have been found outside cells, with significant in vivo evidence for their extracellular activities in adhesion, migration and various other biological processes.¹⁹⁻²² The trafficking routes enabling these annexins to exit cells are not fully

understood and several non-conventional secretion pathways have been discussed.^{10,11,20-23} In contrast to the aforementioned annexins, very few studies have implicated extracellular AnxA6 locations. Probably unrelated to cell migration, AnxA6 was identified as a cell surface receptor for β -glucuronidase on liver membranes.³⁹ In ER-positive and ErbB2-amplified SKBR3 breast cancer cells, Ca^{2+} influx coupled to the interaction of AnxA6 with S100A8 and S100A9 proteins may facilitate translocation of AnxA6 from the inner to the outer leaflet of the plasma membrane, and is possibly relevant in neutrophil chemotaxis and adhesion as well as inflammation-related cancers.⁴⁰ Alternatively, in BT-549 breast cancer cells, it was proposed that AnxA6 and other annexins are secreted into exosomes via lysosomal exocytosis to contribute to focal adhesion organization and invasiveness (see above).³⁰

Indeed, exosomes carry a diverse set of bioactive molecules that can promote cell adhesion and motility⁴¹ and within those from smooth muscle cells, AnxA6 was also identified.⁴² In fact, in the publicly available ExoCarta exosome database (www.exocarta.org), several annexins, including AnxA6, belong to the top 100 proteins often identified in exosomes. The mechanisms that could translocate AnxA6 into the lumen of exosomes are still unclear, but AnxA6 and several other annexins contain a KFERQ-like motif that facilitates chaperone-mediated autophagy, thereby translocating cytosolic proteins inside late endosomal/(pre-)lysosomal vesicles.⁴³ In fact, secretion of proteins via exosomes starts by accumulation in this compartment, followed by their transport and fusion with the plasma membrane. Autophagy and exosomes are increasingly recognized as determining factors for integrin trafficking and focal adhesion turnover, promoting cancer cell motility.^{41,44,45} Most excitingly, recent proteomic studies on the signature of exosomes from cancer-associated fibroblasts identified AnxA6 complexed with LDL receptor-related protein 1 and thrombospondin-1 to support pancreatic cancer aggressiveness (Table 1).⁴⁶ These findings suggest that cellular export of AnxA6 through this route contributes to affect migratory cell behavior. Moreover, AnxA6 containing exosomes were restricted to serum from pancreatic ductal adenocarcinoma (PDA) patients, providing potential for AnxA6 to become a therapeutic target and marker for PDA grade.⁴⁶

Additionally, in a number of cell and animal models, AnxA6 and several other annexins were shown to be vital for membrane repair.⁴⁷⁻⁵¹ In zebrafish and live muscle fibers from mice, AnxA6 is rapidly recruited to damaged sarcolemma to contribute to the formation of a tight cap over a vesicle-rich repair zone.⁴⁷⁻⁴⁹ In

Table 1. AnxA6 interactions relevant in cell migration and adhesion.

Annexin A6	Interaction	Function	Site of action	References
Extracellular matrix, Exosomes	Glycosaminoglycans (e.g., chondroitin sulfate, heparin, heparin sulfate)	Promote cancer cell adhesion	Extracellular	31-33
	Fetuin-A	Promote cancer cell adhesion and motility	Extracellular	30, 35-37
	S100A8, S100A9	Neutrophil chemotaxis and adhesion, inflammation-related cancers	Extracellular	40
	LDL receptor-related protein 1 (LRP1), thrombospondin 1 (TSP1)	AnxA6-containing LRP1/TSP1 complexes on exosomes support pancreatic cancer aggressiveness	Extracellular	46
	Dysferlin, actin, AnxA1, A2, A5	Leakage of AnxA6 into extracellular space during membrane repair (?)	Extracellular	47-51
Membrane lipids	Negatively charged phospholipids and cholesterol in cholesterol-rich domains	Spatial microdomain organization for cell adhesion and migration	Focal adhesions, lipid rafts (incl. caveolae)	9, 54, 63
	Negatively charged phospholipids in non-raft domains	Receptor-mediated endocytosis	Clathrin-coated pits	9, 54
Ca ²⁺ - signaling	Two pore channel 1/2 (TPC1/2) Ryanodine receptors	Ca ²⁺ -channels to regulate Ca ²⁺ -influx during cell migration	Late endosomes, endoplasmic reticulum	74, 75
Cytoskeleton	Actin	Cytoskeletal rearrangements and formation of reversible membrane-cytoskeleton complexes	Cortical cytoskeleton at cell protrusions, membrane and circular-dorsal ruffles	12, 13, 25, 67-70
	α -actinin	Actin-dependent regulation of calcium entry	Cell leading edge	72
	Spectrin, Adaptor protein complex 2 (AP2)	Recruitment of calpain to cleave spectrin-actin cytoskeleton for endocytic trafficking of integrins, receptors, signaling proteins	Plasma membrane, endosomes	59, 78-80
	Epidermal growth factor receptor (EGFR), protein kinase C α (PKC α)	Scaffold function: PKC α recruitment for EGFR inactivation to enable focal adhesion turnover? PKC α recruitment to focal adhesions to regulate integrin trafficking?	Specialized sites at plasma membrane (incl. focal adhesions, caveolae), endosomes (cholesterol rich?)	24, 25, 38
Signaling proteins	H-Ras, p120GAP, Raf-1	Scaffold function: p120GAP recruitment for Ras inactivation p120GAP/p190RhoGAP interaction? Scaffold function?	Specialized sites at plasma membrane (incl. focal adhesions, caveolae), endosomes (cholesterol-rich?)	24-26, 28, 84
	Pyk2, Lck, Fyn	Control of LE-cholesterol export to regulate:	Focal adhesions?	9, 25, 91, 92
	Cholesterol-enriched late endosomes (LE), LE-cholesterol transporters (e.g., Niemann Pick C1), other LE proteins: Ca ²⁺ -channels, Tre-2/Bub2/Cdc16 family member 15 (TBC1D15)?	(1) Caveolin transport and caveolae formation (2) Synaptosomal-associated protein 23/syntaxin-4 dependent fibronectin secretion (3) Syntaxin-6 dependent integrin recycling	LE-cholesterol delivery to the Golgi, recycling endosomes and plasma membrane	24, 25, 27, 29, 65, 116, 118-120

addition to membrane repair in muscle, AnxA6 also contributes to restore membrane lesions induced by bacterial pore-forming toxins.^{50,51} It is tempting to speculate that during sarcolemma cap formation, outward vesiculation or shedding for the removal of toxin-induced pores⁴⁷⁻⁵¹ some AnxA6 proteins might 'leak' into the extracellular space. Although in vivo evidence for extracellular AnxA6 activity has yet to be provided, these opportunities for protein leakage into the extracellular milieu may transiently create AnxA6-enriched local microenvironments with consequences for cell adhesion and migration (Table 1). In fact, elevated AnxA6 levels in patient serum has recently been proposed as a marker for esophageal adenocarcinoma, an aggressive cancer type.⁵²

Annexin A6 and remodeling of plasma membrane domains

At the plasma membrane, the spatial organization of specific proteins and lipids into discrete regions creates microdomains with distinct signaling functions. This includes focal adhesions at the leading edge, where the recruitment and activation of integrins has been linked to cholesterol-rich membrane domains (lipid rafts).^{5,6,53} In line with a function in microdomain formation, relevant for focal adhesions, AnxA6 binds cholesterol-enriched (ordered) membranes in vitro, translocates to cholesterol-rich and detergent-resistant membranes (lipid rafts) upon Ca²⁺ elevation and is enriched in cholesterol-rich caveolae, a specialized type of raft

(Table 1).⁵⁴ In particular caveolae and its structural protein caveolin have been associated with directional migration and invasion, coordination of Rac/Rho activation, focal adhesion turnover, and enhanced metastatic potential in advanced stages of some cancers.⁵⁵⁻⁵⁸ On the other hand, AnxA6 also localizes to non-raft (fluid) domains rich in negatively charged phospholipids such as clathrin-coated pits (Table 1).⁵⁹ These structures facilitate endocytosis of growth factor receptors such as the EGFR, which signals from endosomal compartments to accelerate wound re-epithelialisation, and promote cell migration and angiogenesis.^{9,60} In addition, integrins are also endocytosed via clathrin-coated pits, which is critical for integrin recycling and focal adhesion turnover during directional migration.^{2,3,61} Yet, although the membrane-binding properties of annexins had long been proposed to organize membrane domains, evidence from live cells that AnxA6 or any other annexin could alter microdomain properties, was missing.

We recently addressed this critical issue in the annexin field by labeling cells with the fluorescent probes di-4-ANEPPDHQ and laurdan, which enables visualization of liquid-ordered (cholesterol-rich, raft-like) and liquid-disordered (cholesterol-poor, non-raft) microdomains at the plasma membrane.⁵⁴ This approach enabled comparison of membrane order in AnxA6-deficient and AnxA6 expressing A431 cells^{26,34} as well as mesenchymal mouse embryonic fibroblasts from wild-type and AnxA6 knockout mice.⁶² Strikingly, significant changes in membrane order, reflecting lipid re-distributions at the plasma membrane, and increased sensitivity of membrane order toward cholesterol-depleting agents were observed in cells with elevated AnxA6 levels.⁵⁴ Likewise, analysis of laurdan-stained T cells isolated from WT and AnxA6^{-/-} mice supported AnxA6 to alter the membrane environment in the plasma membrane.⁶³ From these observations we predicted consequences in the distribution of cell surface proteins. Indeed, PALM super-resolution microscopy identified association of AnxA6 up/downregulation with differential and cell-specific differences in the cholesterol-sensitive microdomain distribution of membrane-anchored, fluorescent raft and non-raft marker proteins.⁵⁴

In spite of this, what are the underlying mechanisms that enable AnxA6 to alter microdomain formation? The well-established Ca²⁺-inducible AnxA6 translocation to the plasma membrane is probably one critical aspect to alter phospholipid and cholesterol distribution and organization. Here, AnxA6 preferentially binds negatively charged phospholipids (phosphatidylserine, phosphatidylinositol, phosphatidic acid), and has some affinity toward phosphatidylethanolamine and arachidonic acid.^{24,25} In addition, acidic pH and Ca²⁺-independent

affinity to cholesterol-rich membranes also contributes to AnxA6 membrane association (see above).^{54,64,65} Although limited resolution of the overlay of AnxA6 immunostaining with laurdan staining did not yet enable us to identify a preference of AnxA6 translocation to raft or non-raft microdomains at the plasma membrane (Grewal, Rentero and Enrich, unpublished), it is likely that the recruitment of AnxA6 to specific sites will be determined by the differential affinity toward these various lipids, localized changes in Ca²⁺ and pH, as well as membrane-bound protein binding partners (see below). This could possibly stabilize microdomains for transient signaling events. In turn, once recruited to the membrane, pools of AnxA6 in specialized domains, for example focal adhesions, seem to contribute to induce lipid re-distributions and recruit signaling proteins (see below) to create microenvironments that allow signal transduction and actin-re-arrangements for dynamic focal adhesion assembly and disassembly.

Annexin A6 - actin interactions contributing to domain formation

Besides re-organization of membrane lipids, focal adhesion dynamics are intimately linked to actin re-arrangements as the anchoring of actin filaments is critical for localized transduction of mechanical forces across the plasma membrane for forward movement. In this scenario, bridging molecules like talins and kindlins bind to integrins, and recruit cytoskeletal and signaling proteins.⁶⁶ However, knowledge on proteins that stabilize the association of these multifactorial focal adhesion protein complexes and actin filaments to lipids in membranes is still limited. Hence, the ability of AnxA6 to interact with cytoskeletal proteins has to be considered (Table 1). Indeed, AnxA6 binds actin filaments in a Ca²⁺-dependent manner^{12,13,25,67} interacts with α -actinin,⁶⁸ and co-localizes with F-actin at protrusions of the plasma membrane, including membrane ruffles,^{25,67,69} and circular-dorsal ruffles (Reverter and Enrich, unpublished), that are essential for directed cell motility, cell spreading and adhesion.⁷⁰

Ca²⁺-dependent binding of AnxA6 to pointed ends of actin filaments and α -actinin could contribute to form reversible, membrane-cytoskeleton complexes.^{67-69,71} In cells labeled with laurdan, membrane order of AnxA6 expressing cells showed an increased sensitivity toward actin depolymerizing agents.⁵⁴ Moreover, we observed F-actin accumulation at the plasma membrane upon expression of membrane-anchored AnxA6.⁷² These striking reorganizations of cortical actin correlated with a role for AnxA6 in the maintenance of intracellular Ca²⁺ homeostasis via actin-dependent regulation of

Ca^{2+} entry at the cell surface, with consequences for cell proliferation.⁷² Given that lower Ca^{2+} levels at the leading edge are required for cell migration⁷³ and the fact that AnxA6 interacts with other Ca^{2+} channels, including two pore channel 1/2 and ryanodine receptors in late endosomes (LE) and the endoplasmic reticulum,^{74,75} one can contemplate that besides direct interactions of AnxA6 with cytoskeletal proteins, the ability of AnxA6 to modulate Ca^{2+} influx and homeostasis participates in regulating cytoskeletal re-arrangements in cell migration (Table 1).

In addition, upon Ca^{2+} -mobilization, AnxA6 interacts with spectrin to trigger the recruitment of calpain I, which cleaves the spectrin-actin cytoskeleton, allowing the entry of endocytic vesicles at the plasma membrane.⁵⁹ Interestingly, calpain is a ubiquitous molecule and alternative mechanisms of calpain activation, including Ca^{2+} elevation, have also been proposed. Moreover, dissection of calpain-mediated proteolysis identified many targets in adhesion complexes, including talin, paxilin, α -actinin, ezrin, FAK and the cytosolic tails of $\beta 1$ and $\beta 3$ integrins. Additionally, calpain-mediated cleavage of ezrin is critical to limit fibroplasia late in the regenerative phase of skin repair.^{76,77} Altogether, these findings intimately link calpains with the integrin signal transduction machinery driving cell motility.^{76,77}

In support of AnxA6 being involved in the generation of endocytic vesicles, possibly through spectrin/calpain-dependent mechanisms^{59,78} AnxA6 stimulated vesicle budding in vitro,⁷⁹ and bound to the μ -subunits of the clathrin adaptor complex AP2 in a Ca^{2+} -dependent manner.⁸⁰ These findings correlate with spectrin modulating integrin motility at the cell surface for adhesion and ECM degradation. In addition, loss of spectrin impairs cell adhesion and spreading, alters actin organization and focal adhesions, and modifies expression of integrins.^{81,82} In line with these findings, the selective endocytosis of α integrins appears to be driven by their interaction with AP2.⁸³ Hence, AnxA6-mediated reorganization of the spectrin cytoskeleton might contribute to allow the internalization and recycling of integrins, but also growth factor receptors, signaling proteins like Src and FAK at the plasma membrane for focal adhesion turnover. One can even speculate that through its transient membrane binding behavior, AnxA6 may provide a platform for simultaneous, dynamic and reversible interactions with both actin and spectrin at the plasma membrane (Table 1).

AnxA6 and recruitment of signaling proteins

In addition to AnxA6 re-organizing membrane lipids and possibly serving as a membrane anchor for the actin

cytoskeleton to transiently create and stabilize membrane microenvironments, a major scaffold function of AnxA6 is to facilitate localized recruitment of signaling proteins. AnxA6 binds to a panel of signaling proteins^{8,9,24,25} and some of those interaction partners have prominent roles in cancer cell migration (Table 1). In particular, AnxA6 interacts constitutively with PKC α and p120GAP in the cytosol, both negative regulators of the EGFR and Ras/mitogen activated protein kinase (MAPK) pathway.^{24-26,28,38} Upon EGFR activation, AnxA6 promotes the Ca^{2+} -dependent membrane recruitment of PKC α and p120GAP, leading to increased EGFR/PKC α and H-Ras/p120GAP assembly and consequently, EGFR and H-Ras inactivation. At the plasma membrane, AnxA6 probably stabilizes EGFR/PKC α and p120GAP/H-Ras complexes, as AnxA6 co-precipitates with EGFR in a Ca^{2+} -inducible fashion and interacts with active (H-Ras-GTP), but not inactive H-Ras (H-Ras-GDP), respectively.^{24-26,28,38} Together with AnxA6 co-purifying with Raf-1,⁸⁴ the scaffold function of AnxA6 likely involves multiple simultaneous protein-protein interactions, stabilizing compartmentalized multifactorial protein complexes that ensure EGFR and Ras signal termination.

In our previous studies, we solely investigated these interactions in the context of cancer cell growth. Indeed, we observed anti-proliferative properties of AnxA6 via PKC α - and p120GAP-mediated EGFR and H-Ras down-regulation in EGFR overexpressing A431, BCCs and HNSCCs as well as ER-negative BCCs.^{24-26,28,38} This is supported by earlier reports from Moss and coworkers⁸⁵ and our unpublished data showing reduced xenograft growth of A431 cells ectopically expressing AnxA6. Vice versa, increased growth of MDA-MB-231 and HeLa cell lines upon AnxA6 depletion was observed.^{28,38} Altogether, these findings implicate AnxA6 as a tumor suppressor to inhibit growth of cancer cells through EGFR and Ras inactivation.^{24,25} However, there is also substantial literature that points to the prominent crosstalk of de-regulated EGFR and Ras activity with integrin, FAK, Src, Rac/Rho GTPases signaling in cancer cell metastasis. It would go beyond the scope of this article to describe these complex networks in more detail and we recommend excellent review articles from others.^{3-5,86-90} In addition, AnxA6 has also been described to interact with Pyk2, and the Src kinases Lck and Fyn^{9,25,91,92} all of which with established links to migratory events in cancer cells.⁸⁷ Indeed, AnxA6 overexpression in A431 and Chinese hamster ovary (CHO) cells reduced migration in scratch- and transwell assays, which correlated with reduced invasion in 3D organotypic assays (Fig. 1) and strongly reduced activation of FAK and Src.²⁹ In line with this, AnxA6 depletion in HeLa and MDA-MB-231

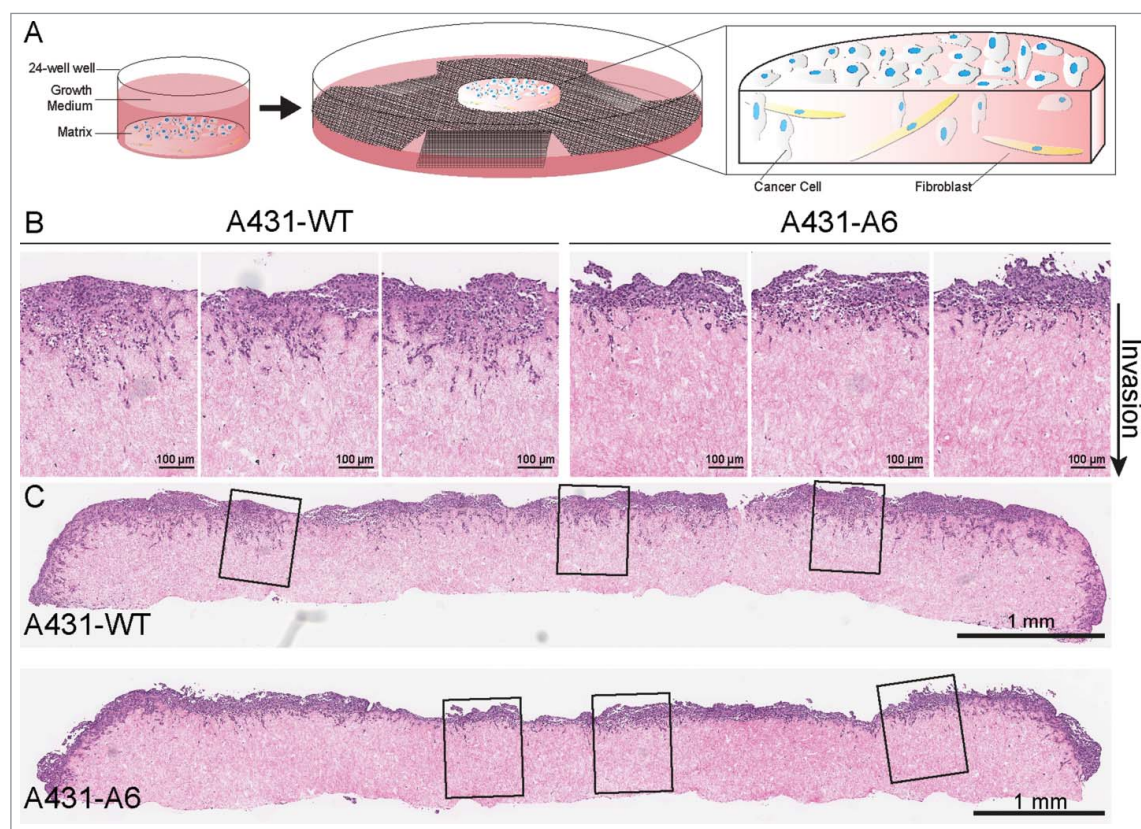


Figure 1. A 3-dimensional organotypic invasion assay highlighting the reduced invasiveness of AnxA6 expressing A431 cells. (A) The scheme shows an organotypic invasion assay for the three-dimensional assessment of tumor cell behavior. Primary human fibroblasts are embedded in a 3D collagen-I matrix, prepared from acid extracted rat tail collagen. Detached, polymerized matrix is allowed to contract for 12 d in complete media, until embedded fibroblasts have contracted the matrix to ~1 cm in diameter. This forms a matrix with high *in vivo* fidelity. Tumor cells are then plated on top of the matrix in complete media and allowed to grow to confluence for ~3–5 d. The matrix is then mounted on a metal grid, raising it to an air/liquid interface, which results in a chemoattractive gradient feeding the matrix from below. Media is changed every 2–3 days, and after 8–21 days, cultures can be fixed, processed (e.g. hematoxylin and eosin staining) and analyzed.^{29,114,116,139} (B) Representative cutout images of AnxA6-deficient A431 wild-type cells (A431-WT) and AnxA6 expressing A431 cells (A431-A6) seeded on 3D-matrices and allowed to invade over an air-liquid interface for 12 days, prior to fixation and hematoxylin and eosin staining. The direction of invasion is indicated. Scale bars represent 100 μm. The reduced ability of A431-A6 cells to invade the matrix is apparent (for quantification see also ref. 29). (C) Representative images of whole matrix sections, demonstrating reduced invasion of AnxA6 expressing A431 cells. The enlarged sections shown in B are indicated. Scale bars represent 1 mm.

cells increased. wound closure and transwell migration/invasion.²⁹ Moreover, in wound-healing assays, upregulated AnxA6 levels in A431 cells were associated with increased potency of tyrosine kinase inhibitors targeting EGFR, including gefitinib and erlotinib, to inhibit cell migration (Hoque and Grewal, unpublished). It remains to be determined if these observations are also relevant *in vivo* or in other metastatic cancer models, where oncogenic EGFR and Ras activity are common and AnxA6 downregulation has been observed.^{9,28,93}

Given that signaling proteins at the plasma membrane are not randomly distributed, but targeted and localized to specific microdomains, AnxA6 may promote PKC α - and p120GAP-mediated EGFR/Ras inactivation at specialized sites critical for cell motility. In this context, the spatial distribution of Ras isoforms, small GTPases that

oscillate between an active GTP-bound and inactive GDP-bound form, has been studied intensively.⁹⁴ Active and inactive Ras proteins are organized in nanoclusters, containing 6–7 Ras proteins per nanocluster, creating the signaling platforms essential for the recruitment of Ras regulators and effectors. The distribution and lateral movement of active and inactive Ras proteins in the plasma membrane is modulated by GTP/GDP exchange, thus depends on the activity of guanine nucleotide exchange factors and GAPs. In addition, interaction of membrane lipids with Ras proteins is critical for cluster formation. While H-Ras GDP clusters are mainly found in cholesterol-rich domains, H-Ras-GTP nanoclusters are associated with disordered and cholesterol-independent domains.⁹⁴ Hence, the distribution of cholesterol at the plasma membrane is highly relevant for the

recruitment of H-Ras regulators such as p120GAP, but possibly also for membrane translocation of PKC to phosphorylate and regulate K-Ras activity⁹⁵ and consequently for signal termination. In addition, a distinct cohort of phospholipids, in particular phosphatidylserine, but also phosphatidic acid and phosphatidylinositides contribute to the spatial segregation of Ras isoforms. In line with the constant re-organization of membrane domains at the leading edge, the GDP/GTP cycle of Ras proteins ensures the transient nature of Ras nanoclusters. Interestingly, single molecule image analysis revealed that once recruited to the plasma membrane, the Ras binding domain of p120GAP became transiently immobile to interact with H- and K-Ras-GTP.⁹⁶ Hence, the binding affinity of AnxA6 toward the abovementioned phospholipids, but also cholesterol, might stabilize the recruitment and 'immobilize' p120GAP to active Ras-GTP clusters.

The ability of AnxA6 to act as a membrane targeting protein for p120GAP and being relevant for cell migration may indeed go beyond Ras inactivation. For instance, p120GAP interaction with p190-RhoGAP, FAK, the calpain subunit Capn1 and even integrins has been implicated in the regulation of Rho GTPase activity, Rho-mediated cytoskeletal rearrangements and integrin trafficking.^{93,97-103} Thus, AnxA6-mediated recruitment of p120GAP could coordinate pathways to promote actin remodeling for the formation of focal adhesions, establishment of cell polarity, as well as integrin recycling.^{93,97-103}

In contrast to the advanced knowledge on Ras signaling platforms, the regulation of EGFR distribution at the cell surface is less clear, with substantial amounts of EGFR being found in non-raft domains, but also in cholesterol-rich microdomains.^{9,104} These possibly distinct pools of EGFR seem to provide opportunity for signal diversity, with substantial cross-talk between EGFR, integrins, FAK, Src, and the actin cytoskeleton to integrate the signals required for cell migration.^{9,86-90,104} Possibly AnxA6-mediated stabilization of EGFR/PKC α containing signal complexes, followed by EGFR inactivation contributes to downregulate EGFR-mediated signaling networks required for focal adhesion turnover during forward cellular movement.

Based on the development of PKC α -dependent signaling pathways upon AnxA6 overexpression in cells that do not express EGFR,¹⁰⁵ AnxA6-mediated membrane targeting of PKC α may also be relevant in migratory cell behavior unrelated to EGFR. Many studies have implicated stimulatory roles for PKC α in cancer cell motility and tumor cell invasion and metastasis. For example, PKC α is recruited to cholesterol-rich domains as well as focal adhesions in a Ca²⁺-dependent manner to associate with β 1-integrins and control integrin

trafficking, focal adhesion turnover for cell spreading and polarized cell trafficking.¹⁰⁶⁻¹⁰⁹ PKC α also phosphorylates ezrin and thereby contributes to the accelerated motility of cancer cells.¹¹⁰

Taken together, several cellular activities listed above implicate PKC α , but also p120GAP, to stimulate cell migration. Thus, the proposed model of AnxA6 inhibiting migratory behavior via PKC α and p120GAP-mediated EGFR/Ras inactivation may only be relevant in certain susceptible cell types with high AnxA6 levels, or in the context of co-operating genetic lesions such as EGFR overexpression. Indeed, in BT-549 cells, which express only moderate levels of AnxA6 and EGFR,²⁸ AnxA6 depletion promoted EGFR degradation, attenuated MAPK signaling, reduced cell motility and invasiveness and sensitized BT-549 cells to EGFR tyrosine kinase inhibitors.^{30,111} Likewise, although our recent studies suggested that the absence of AnxA6 in T-lymphocytes destabilized the organization of the plasma membrane, T-cell migration and signaling was not affected in AnxA6-deficient T-lymphocytes.⁶³ Also, in chick mid-brain neural crest cell development, AnxA6 depletion attenuated, while AnxA6 overexpression enhanced neural crest cell emigration, respectively.¹¹² However, opposing cellular activities as observed for AnxA6 are not uncommon, as loss of another scaffold protein, caveolin-1 (Cav-1), predicts poor clinical outcome in some cancers, while its overexpression is linked to aggressive and metastatic behavior in others.⁵⁵⁻⁵⁸ Interestingly, as discussed below, the subcellular localization and ability of Cav-1 to form caveolae is strongly influenced by high/low AnxA6 expression levels in EGFR overexpressing A431, MDA-MB-468 and ER-negative MDA-MB-436 cells,²⁷ yet if these regulatory networks operate in other cells and tissues to possibly modulate cell migration has still to be determined.

The regulatory role AnxA6 in cholesterol homeostasis determines migratory behavior

As outlined above, the function of cholesterol as a membrane constituent comprises several decisive features relevant for the spatiotemporal coordination of signaling and trafficking events during cellular movement. As such, cholesterol depletion at the cell surface disrupts the integrity and dynamics of focal adhesion (dis-) assembly, caveolae formation, the recruitment and recycling of integrins, FN-dependent cell migration, and Rac/Rho GTPase activation.^{1-5,53-58,113,114} Despite these observations, the cellular cholesterol pools delivered into focal adhesions and linking cholesterol metabolism with migratory behavior are not fully known. Rather than synthesizing cholesterol de novo, cells generally acquire

cholesterol through low density lipoprotein (LDL) receptor-mediated endocytosis.^{114,115} Internalized and esterified LDL-cholesterol finally reaches LE/(pre-) lysosomes, where LDL-derived cholesteryl esters are hydrolyzed. LE are the major sorting station for LDL-cholesterol, which is mainly delivered to the plasma membrane, or transported to the endoplasmic reticulum for feedback control or stored as cholesteryl esters in lipid droplets. Using Niemann Pick Type C1 (NPC1) mutant cells, which accumulate cholesterol in LE, we and others recently demonstrated that LE-cholesterol is crucial for cancer cell migration.^{116,117} The underlying mechanisms involve compromised LE-cholesterol delivery to the plasma membrane, trans-Golgi network and recycling endosomes, as reduced cholesterol levels in these compartments interfered with focal adhesion and caveolae numbers, FN secretion and integrin recycling, with drastic effects on migratory cell behavior.¹¹⁶⁻¹¹⁹

Most strikingly, in addition to AnxA6 directly affecting membrane organization, the actin cytoskeleton and recruitment of signaling proteins at the plasma membrane, we recently identified AnxA6 to indirectly regulate migratory behavior through inhibition of LE-cholesterol distribution (Table 1).²⁹ Using LDL or the amphipathic steroid U18666A, we initially identified translocation of AnxA6 proteins to cholesterol-enriched LE.^{65,120} Moreover, elevated AnxA6 levels led to an NPC1-like phenotype, characterized by LE-cholesterol accumulation and a concomitant diminution of cholesterol at the plasma membrane, Golgi apparatus, and recycling endosomes.^{24,25,27} In several studies we demonstrated that the cholesterol imbalance induced by AnxA6 (i) perturbed cholesterol-dependent Golgi recruitment of cytosolic phospholipase A2 to drive caveolin transport to the cell surface^{27,118} but also led to mislocalization of several SNARE proteins: (ii) soluble N-ethylmaleimide-sensitive fusion protein 23 (SNAP23), syntaxin 4 (Stx4),¹¹⁹ and (iii) Stx6,¹¹⁶ all of which with critical roles in cell migration (see Table 1 and below).

First, AnxA6-mediated inhibition of Cav-1 export from the Golgi significantly reduced the numbers of caveolae at the cell surface.²⁷ In fact, high/low AnxA6 levels inversely correlated with caveolae numbers in A431 cells and several BCCs.²⁷ Hence, AnxA6 expression levels could be one of the factors determining the ambivalent roles of Cav-1 in cancer progression. Cav-1 promotes migration in a number of tumor-derived cell lines, yet has also been identified to inhibit cancer cell migration.⁵⁵⁻⁵⁸ These opposite activities may reflect differential cross-talk of Cav-1 with various members of the cellular networks at the cell surface that drive migration and invasion including growth factor receptors, FAK, Src, and Rac/Rho GTPases. Hence, differential AnxA6 levels in various cancers and its

impact on the cholesterol-dependent Cav-1 cell surface delivery could explain some of the diversity observed for Cav-1 modulating signaling events relevant for cancer cell migration and invasion.⁵⁵⁻⁵⁸

Second, high AnxA6 levels interfered with the clustering of SNAREs Stx4 and SNAP23 in cholesterol-rich domains at the cell surface.¹¹⁹ These widely expressed t-SNAREs normally enable docking of secretory vesicles at the cell surface, but accumulated on Golgi membranes of AnxA6 expressing cells. Consequently, Stx4/SNAP23-dependent FN secretion was strongly inhibited in AnxA6 expressing cells and other NPC1-mutant models.¹¹⁹ This LE-cholesterol related AnxA6 attribute could counteract the well-established fact that increased secretion of ECM proteins such as FN strongly support the signaling cascades that promote migration in oncogenic settings.¹²¹ In addition, these findings are in line with earlier studies that suggested an inhibitory role for AnxA6 in the secretory pathway.¹²²⁻¹²⁴ Thus, AnxA6-mediated inhibition of LE-cholesterol egress is likely to also affect other Stx4/SNAP23-dependent trafficking events often de-regulated in cancer cell migration, including $\alpha 5$ integrin recycling, Src and FAK activation at focal adhesions, and membrane type-1 matrix metalloproteinase delivery to invadopodia.¹²⁵

Third, the most compelling evidence that AnxA6 inhibits cell migration via the establishment of a cellular cholesterol imbalance came from our recent studies that identified AnxA6 overexpression to interfere with Stx6 localization and function,²⁹ a SNARE protein essential for the cell surface delivery of integrins, FAK signaling in focal adhesions, and directional migration toward FN.^{126,127} Strikingly, elevated AnxA6 levels caused Stx6 and integrin accumulation in recycling endosomes (Fig. 2)²⁹ leading to decreased cell surface expression of $\alpha V\beta 3$ and $\alpha 5\beta 1$ integrins, reduced cell migration in wound healing, trans-well and 3D-migration/invasion assays (Fig. 1).²⁹ In line with these findings, individual cell tracking and cell spreading in AnxA6 expressing CHO and A431 cells was inhibited in a Stx6- and NPC1-dependent manner (Fig. 3). Several studies identified Stx6 to be upregulated in breast, liver, and prostate cancers,¹²⁷ and Stx6 elevation to correlate with metastatic esophageal squamous cell carcinoma.¹²⁸ With Stx6 overexpression promoting cell migration,¹²⁷ it remains to be determined if AnxA6 upregulation in these cancers could downregulate oncogenic roles of Stx6. Also, based on the findings described above, AnxA6 upregulation may not only affect Stx4/SNAP23 and Stx6, but also localization and functioning of other cholesterol-sensitive SNAREs.^{129,130} This includes VAMP2, which participates in $\alpha 5$, $\alpha 3$, $\beta 1$ integrins and FAK trafficking¹²⁵ as well as Stx12-dependent Src and $\beta 1$ integrin transport to cholesterol-rich invadopodia for cell invasion.¹³¹

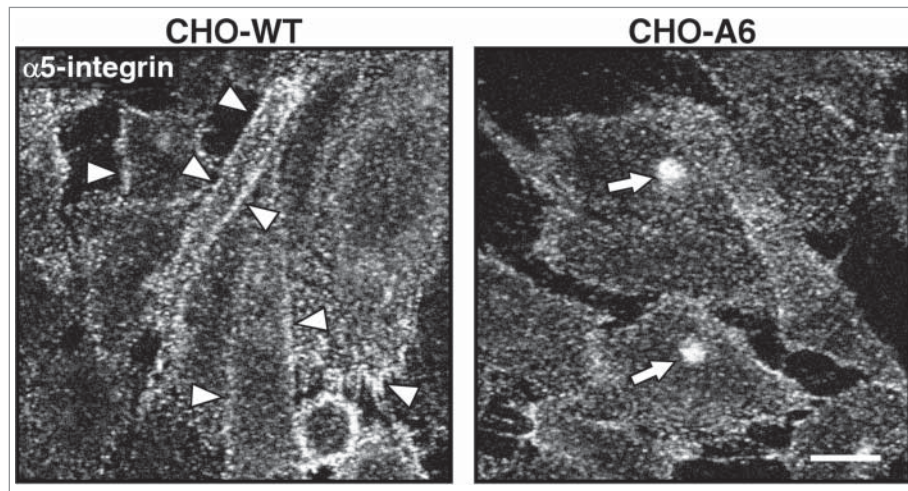


Figure 2. Reduced cell surface expression of $\alpha 5$ integrins in AnxA6 overexpressing CHO cells. (A) Chinese hamster ovary wild-type (CHO-WT) and Annexin A6 overexpressing CHO cells (CHO-A6) were grown on coverslips, fixed with 4% paraformaldehyde and immunolabeled with anti- $\alpha 5$ integrin as described.^{29,116} Arrowheads in control CHO-WT cells show strong staining of $\alpha 5$ integrins at the plasma membrane. Arrows point at the compact staining of $\alpha 5$ integrins in intracellular compartments, resembling recycling endosomes in CHO-A6 cells (for a more detailed characterization of $\alpha 5$ integrin distribution in CHO-WT and CHO-A6 cells see ref. 29). Scale bar is 10 μm .

In strong support of elevated AnxA6 levels interfering with Cav-1, SNAP23/Stx4 and Stx6 functions via perturbed LE-cholesterol transport routes, ectopic NPC1 expression in AnxA6 expressing cells diminished LE-cholesterol accumulation, but also restored cellular distribution of Cav-1, SNAP23/Stx4 and Stx6, with a concomitant elevation of cell surface integrins.^{27,29,119} Despite this approach rescuing the NPC1-mutant like phenotype of AnxA6 expressing cells, and also restoring cell velocity and spreading (Fig. 3), it remains to be identified how AnxA6 interferes with LE-

cholesterol export. AnxA6 binds to cholesterol-rich membranes in vitro and cell culture^{25,54} and a tryptophan residue (W343) within the AnxA6 linker region was proposed important for AnxA6-cholesterol interactions.¹³² More recently, proteome-wide mapping also identified AnxA6 as a potential cholesterol-binding protein.¹²⁹ These findings might reflect AnxA6 affinity to cholesterol-rich rafts and/or caveolae observed in many studies,^{24,25,54} and earlier work from our laboratory, which identified AnxA6 recruitment to cholesterol-enriched LE.^{65,120} We initially proposed that

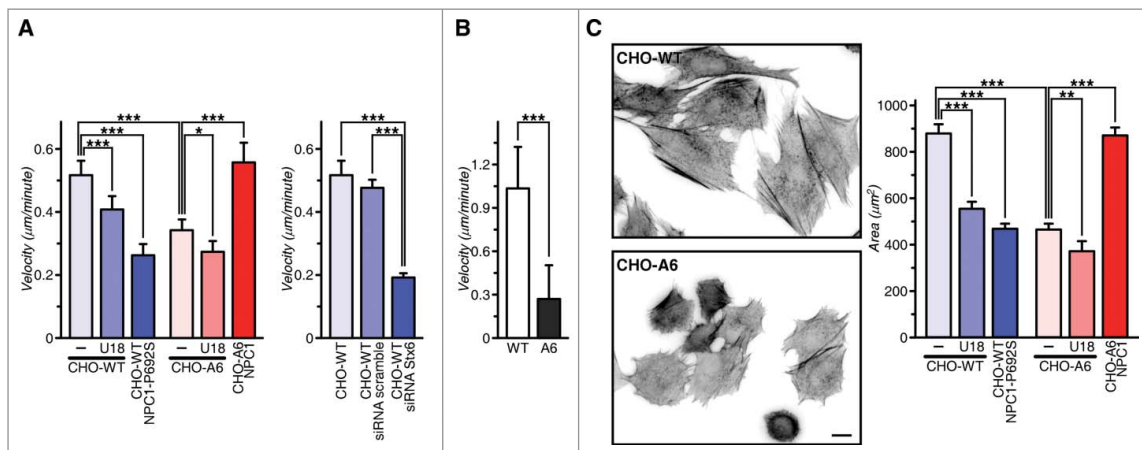


Figure 3. AnxA6 overexpression reduces cell velocity and spreading. (A) CHO-WT, CHO-A6, CHO-WT expressing scrambled or Stx6-targeting siRNA and (B) A431-WT and A431-A6 cells were plated on fibronectin and movement of cells was recorded by time-lapse video microscopy for 12 h. Individual cell migration tracks were marked and the speed (velocity) \pm U18666A (U18), overexpression of wild-type NPC1 in CHO-WT, or dominant-negative NPC1-P692S mutant, which cannot bind cholesterol^{29,116} in CHO-A6 was recorded. (C) CHO-WT and CHO-A6 cells plated onto fibronectin were stained for F-actin after 2 hours. The spreading area (μm^2) \pm U18666A, overexpression of wildtype NPC1 in CHO-WT, or dominant-negative NPC1-P692S mutant in CHO-A6 was determined. Representative fields of both cell lines are shown. 100 cells per experiment were quantified. Scale bar is 10 μm . *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Error bars shown in this figure represent SD.

AnxA6 interfered with NPC1 activity²⁷ but several other cholesterol export pathways from this compartment exist,^{114,115,130} and recent work from our laboratories identified that AnxA6 interacts with a member of the large Tre-2/Bub2/Cdc16 protein family, TBC1D15, a Rab7-GTPase activating protein (Enrich, Grewal and Rentero, unpublished). Moreover, preliminary studies suggest that AnxA6 recruits TBC1D15 to LE, which inhibits Rab7 GTPase activity and its ability to promote LE-cholesterol export (Enrich, Grewal, and Rentero, unpublished). These findings link AnxA6 and LE-cholesterol transport with increasing evidence that associate this member of the Rab protein family with actin-dependent cell migration and metastasis,¹³³⁻¹³⁵ as well as the translocation of integrins, Src kinase and metalloproteases from LE to focal adhesions.¹³⁶⁻¹³⁹

Conclusion

In summary, multiple avenues for AnxA6 being involved in cell motility have emerged and have been listed in Table 1: (1) Interaction of extracellular AnxA6 proteins with ECM proteins may affect adhesive and migratory cell properties; (2) AnxA6 translocation to the plasma membrane provides scaffolding functions that lead to lipid and actin cytoskeleton re-arrangements, and enable recruitment of signaling proteins with consequences for focal adhesion assembly and signaling; (3) AnxA6-mediated inhibition of LE-cholesterol export causes cellular cholesterol imbalance that interferes with multiple Cav-1 and SNARE functions required for cellular movement. In fact, the latter function of AnxA6 listed here (see (3) above) might emerge as an overarching theme how cholesterol homeostasis coordinates the multiple involvements of AnxA6 in cell motility and adhesion. Recruitment of AnxA6 to cholesterol-rich late endosomes will most likely govern the amounts of AnxA6 that translocate into exosomes. This could be a crucial factor determining the levels of secreted AnxA6 and its impact on migration and adhesion (see (1) above). In addition, the ability of AnxA6 to control LE-cholesterol export critically affects microdomain formation at the cell surface. While this may not directly impact on constitutive, growth factor or Ca^{2+} -induced AnxA6 interactions with signaling proteins in the cytosol, it could be decisive to define membrane recruitment and targeting of these complexes to specific sites in specialized microdomains (see (2) above). In this manner, up- or downregulation of a relatively small pool of AnxA6 proteins in LE through metabolic adaptation of cholesterol amounts delivered to this compartment would control the localization and activity of the total pool of AnxA6 proteins throughout the cell. Understanding how the scaffolding and cholesterol transport functions of AnxA6 are coordinated will ultimately contribute to a better understanding of how cellular cholesterol distribution links with domain

formation, actin dynamics and signaling complex formation in cellular events driving cell migration and invasion.

Given that many cancer cells, including A431 cells, show elevated LDL receptor levels and increased LDL uptake,^{114,139-141} AnxA6 expression levels could become a determining factor that permits or inhibits scenarios enabling Cav-1, Stx6, Stx4 and SNAP23 to promote integrin recycling, ECM cell surface delivery, or aberrant Src and FAK signaling. Furthermore, AnxA6 expression in A431 cells inhibited EGFR/Ras via PKC α /p120GAP pathways,^{26,28,38} while AnxA6-induced cholesterol imbalance triggered Cav-1, SNAP23/Stx4 and Stx6 mislocalization to reduce caveolae formation, FN secretion and integrin recycling in these cells.^{27,29,119} Hence, the cooperative and simultaneous involvement of multiple AnxA6 properties in different cellular sites could decrease the ability of A431 cells, and possibly other cancer cells, to migrate and invade in 2- and 3-dimensional environments. Given the increased complexity of cell and animals models available, future studies will have to aim and dissect the contribution of the scaffolding and cholesterol transport functions of AnxA6 and their relative contribution to metastatic behavior.

Abbreviations

AnxA6	annexin A6
ARFs	ADP ribosylation factors
BCC	breast cancer cells
Ca^{2+}	calcium
Cav-1	caveolin-1
CHO	Chinese Hamster Ovary
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
ER	estrogen receptor
Erk	extracellular signal-regulated kinase
FAK	focal adhesion kinase
FN	fibronectin
HNSCCs	head and neck squamous cell carcinoma
LDL	low density lipoprotein
LE	late endosomes
MAPK	mitogen-activated protein kinase
NPC1	Niemann Pick Type C1
p120GAP	p120 GTPase activating protein
PKC α	protein kinase α
SNAP23	soluble N-ethylmaleimide-sensitive fusion protein 23
SNARE	soluble NSF attachment protein (SNAP) receptor
Stx	syntaxin

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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