A Filamin A Binding Protein Regulates Vimentin Assembly and Cell Extension Formation

By

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Abstract

The actin and intermediate filament cytoskeletons are critical polymer networks that contribute to important pathological processes. Some of these processes involve the generation of cell extensions, which enable remodeling of the extracellular matrix and invasion of the matrix by malignant tumors. Currently the mechanisms by which the actin and vimentin cytoskeletal systems functionally interact to mediate cell extension formation and matrix invasion are not defined. I hypothesized that the actin binding protein filamin A, enables vimentin intermediate filament assembly, thereby enhancing the formation of cytoplasmic extensions. In cultured fibroblasts, shRNA knockdown of filamin A reduced the formation of cell extensions by >4-fold; siRNA knockdown of vimentin resulted in >4-fold shorter cell extensions. Vimentin filament assembly was enhanced in cells expressing filamin A, suggesting a potential functional interaction between these two proteins.

Acknowledgment

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I. Literature Review

In this thesis I focus on defining the signaling system(s) and the intermediate filament and microfilament cytoskeletal interactions that are involved in generating cell extensions. In addition to a large number of other activities (e.g. cell migration), these specialized types of cellular protrusions are organelles that mediate degradation of soft connective tissues by phagocytosis (Everts et al., 1996), an important process in ECM remodeling. In the first part of this literature review I describe in considerable detail the fundamental properties of the ECM, which provides in-depth context for the importance of cell extensions in ECM remodeling. Later, I review the processes by which the ECM is remodeled and the role of cell extensions in these remodeling processes and the proteins that may contribute to the formation and function of these organelles.

A. ECM Components and Structure

The ECM provides support and attachment for organs and enables the appropriate function and maintenance of the structural elements of many tissues (Lu et al., 2011). In addition to its support and structural functions, the ECM physically contributes to the spatial positioning of cells within tissues and isolates cells from adjacent tissue layers, which is exemplified by the basement membrane that separates the basal epithelial cells of the epidermis from the underlying lamina propria of soft connective tissues. The exchange of informational content encoded in matrix molecules through matrix protein adhesion receptors expressed on epithelial and connective tissue cells enables bidirectional signaling (Bissell et al., 1982) that profoundly affects cell function and helps to organ and tissue homeostasis.

Balanced remodeling of the ECM by synthesis and degradation of ECM components preserves the normal structure and function of connective tissues. Remodeling is a dynamic process that is markedly sensitive to environmental perturbations including trauma, infection and the invasion of the ECM by cancer cells. In healthy tissues, the systems that preserve ECM structure and function are critically involved in the appropriate regulation of cell proliferation, cell migration and cell differentiation to maintain tissue homeostasis.

The ECM comprises a large cadre of proteins, glycoproteins, glycosaminoglycans, proteoglycans and carbohydrates, which collectively are part of the "matrisome" as proposed by Richard Hynes and his colleagues (Naba et al., 2016). In this conceptual approach to understanding the interacting functions of various ECM components and maintenance of tissue homeostasis, a bioinformatic approach was developed based on the work of George Martin and colleagues. Martin's group suggested the notion of the "matrisome" in the restricted context of the basement membrane. They defined "supramolecular complexes of matrix components, which are the functional units of the forming extracellular matrix" (Martin et al., 1984). Later work by Hynes and colleagues (Hynes and Naba, 2012; Naba et al., 2012) extended the definition of the matrisome to include not only all the genes that encode structural ECM components (e.g. collagens, proteoglycans, glycosaminoglycans, fibronectins) but in addition, those genes that encode proteins that interact with or remodel ECM components (e.g. matrix-degrading proteases, matrix adhesion receptors, proteins that regulate adhesion receptor function). Hynes and his group screened human and mouse proteomes and employed defining features of ECM proteins (e.g. signal peptide; protein domains suggestive of ECM proteins) as part of the data filtering function in the bioinformatics process. Based on structural or functional features, they separated core matrisome proteins from matrisome-associated proteins.

The core ECM matrisome of animals, which comprises the principal structural components of the ECM (e.g. collagen, fibronectin, hyaluronan, decorin), can be conceptually divided into various domains. One of these domains includes the interstitial matrix, which is comprised of soluble and fibrillar proteins (like nascent collagen molecules and cross-linked, higher order structural arrays of collagen fibers), glycosaminoglycans (like hyaluronic acid), proteoglycans (aggrecan) and glycoproteins (like fibronectin). Across a broad array of different tissues, it is evident that the repertoire of ECM molecules is tissue-specific, such as the proteins in basement membranes (e.g. type IV collagen, laminin, periostin, fibulin) that separate the lamina propria of soft connective tissues from their adjacent, covering epithelia and which provide the support and attachment for the basal epithelial cells of stratified squamous and simple epithelia (Eble and Niland, 2019).

In addition to the core matrisome, ECM-affiliated proteins, ECM regulators and secreted factors are thought to interact with core ECM proteins (Hynes and Naba, 2012). Among these ECM regulators, a large and diverse group of proteins and systems are involved in ECM synthesis, degradation and turnover. These regulators in turn are intimately involved with, or in some cases are comprised of the actin, intermediate filament and microtubular cytoskeletons, which in turn are involved in the transmission and transduction of signals through ECM adhesion receptors and the generation of cell extensions that are needed for ECM degradation.

B. Role of ECM remodeling in health and disease

Consistent with the dynamic nature of ECM components that is observed in many developing tissues, the remodeling of the ECM is functionally involved in several discrete processes in vertebrate organogenesis (such as liver, kidney, heart and lung) and in the morphogenesis of

specialized tissues (such as secretory glands). For example, branching morphogenesis, an essential process in the ontogeny of many organs such as lungs and kidneys, involves groups of cells that generate cell extensions during specific stages of organ development in order to increase the surface area of the developing structure (Lu et al., 2011). The formation of these cell extensions enables cells to locally remodel their surrounding interstitial ECM and thereby to optimize matrix support and attachment for overlying secretory, transport or absorptive epithelial cells.

Disturbances of matrix remodelling in which the normal structure and function of ECM components are dysregulated, is frequently observed in a broad array of fibrotic diseases such as oral sub-mucous fibrosis (associated with betel nut chewing by Tamils in Sri Lanka), idiopathic pulmonary fibrosis, and cardiac hypertrophy and fibrosis secondary to pressure overload (i.e. hypertension). Notably, idiopathic pulmonary fibrosis is a chronic, relentlessly progressive, fatal illness marked by excessive alveolar scarring and increased stiffness of pulmonary tissues, which eventually leads to pulmonary structural damage, greatly reduced oxygen exchange and global loss of lung function (Upagupta et al., 2018).

The synthesis of collagen molecules and their extracellular assembly into higher order structures (collagen fibrils, collagen fibers, cross-linked collagen fiber arrays) are central features of the production of the specialized ECM that is required for normal organogenesis, or in the formation of the diseased matrix in fibrotic lesions or in the repair of damaged tissues in wound healing. The formation of the nascent matrix of soft connective tissues involves several cell types but fibroblasts and their various sub-types in particular have been studied in great depth. These cells synthesize a large number of different types of matrix components, although the most abundant molecule by far is type I collagen (Perez-Tamayo, 1978). Fibroblasts synthesize collagen molecules, assemble these nascent molecules extracellularly

into fibrils and fibers, and then organize and cross-link the fibers into complex and highly structured arrays that comprise a large volume fraction of many soft connective tissues. Fibrillar collagen arrays are indispensable in wound healing (see below) and for the repair of tissue damage after injury. However, in some tissues that are subjected to chronic injury or stress (e.g. the pressure overload of hypertension), there is often the development of excessive and poorly organized interstitial collagen in the left ventricular wall of the heart, which results in myocardial stiffness and impaired diastolic function in the cardiac cycle (Berk et al., 2007). This is an example of a fibrotic lesion in which excessive and poorly organized deposition of fibrillar collagen affects the mechanical properties of the heart wall and which as a result, contributes to the development of heart failure, a high prevalence, high morbidity disease with few effective treatments.

Abnormalities in ECM and collagen remodeling and degradation are also manifest in metastatic processes, which involve the invasion of cancer cells into adjacent, often healthy soft connective tissues. For example, in oral squamous cell carcinoma, ECM components in basement membranes such as type IV collagen, laminin and heparan sulphate proteoglycans, which normally create a dense mesh-work for provision of polarity and rigidity in basement membrane, are degraded by cancer cell-associated proteases. These alterations decrease cancer cell-matrix adhesions and thereby facilitate the invasion of the matrix by extensions formed by tumour cells, allowing these cells to more easily traverse membrane barriers and migrate to blood vessels for creation of metastases (Kumar and Hema, 2019). The progression of breast cancer is also strongly influenced by dysregulated ECM protein networks. In breast cancer, along with increased deposition of a fibrotic matrix, which includes type I, III, V collagens, elastin, elastin, vitronectin, and fibronectin, there is also elevated glycosaminoglycans. In contrast, type IV collagen and laminin LM-111, which are major components of the normal

basement membrane, are degraded, which contributes to the invasiveness of metastatic cancer cells (Oskarsson, 2013).

ECM remodelling is a prominent process in the restoration of normal tissue structure and function that occurs in wound healing after injury. Cutaneous wound healing has been examined in great depth and is comprised of several discrete stages including an early, inflammatory response, the formation of granulation tissue, wound re-epithelialization, angiogenesis, and matrix remodeling. Following the initial inflammatory response, fibroblasts are attracted into the provisional matrix of the wound site where they form granulation tissue. During this phase, type III and type I procollagen, elastin, proteoglycans, and hyaluronic acid are secreted and deposited into the provisional matrix in order to enable the ingrowth of blood vessels that in turn support the expansion of new connective tissues. During wound closure, matrix remodelling processes contribute to the alignment of collagen fibres and the formation of cross-links between adjacent lysine residues in collagen fibers. This cross-linking process, which is mediated by lysyl oxidase and other related enzymes, increases the stiffness of collagen fibers and enables restoration of the tensile strength of the wound (Xue and Jackson, 2015).

C. Mechanisms of ECM Remodeling

To maintain normal tissue structure and function, the molecular components of many ECMs undergo continuous turnover during life. The rates of matrix turnover are age, organ and tissue-specific, as has been nicely illustrated for collagen in different sites in the periodontium (Sodek, 1977). Turnover involves the synthesis of new ECM proteins, the remodeling of these proteins to adjust for alterations of functional needs, and the degradation of effete and damaged ECM components.

There are two major routes by which ECM components (and in particular collagen) are degraded: 1) matrix metalloproteinase-mediated extracellular degradation (Nielsen et al., 2019); 2) intracellular (phagocytic) degradation (Everts et al., 1996). In both of these systems cells in the matrix (such as fibroblasts) form cell extensions that enable the cells to remodel the pericellular matrix by extracellular proteolysis and/or by phagocytosis, which involves intracellular degradation in phagolysosomes. For both processes, the extensions that are generated to enable ECM remodeling exhibit enrichment of proteases, which mediate localized, pericellular matrix degradation.

1. Matrix Metalloproteinases (MMPs)

Here I will briefly describe those MMPs that are involved in the degradation of fibrillar collagen, the most abundant protein of mammals (Perez-Tamayo, 1978). There are at least 28 types of MMPs, which are a family of broadly distributed, zinc-dependent endo-peptidases that cleave a large number of ECM proteins but also other, signaling proteins such as MCP-3 (McQuibban et al., 2000). MMP1 and MMP8, along with the membrane type MMPs (e.g. MT-1 or MMP14; enriched on the membranes of cell extensions (Frittoli et al., 2011)) exhibit the unusual ability to cleave the triple helical structure of fibrillar collagens (i.e. they demonstrate triple helicase activity; (Tam et al., 2004)). In terms of structure, MMPs typically exhibit a pro-peptide sequence, a catalytic metalloproteinase domain with a catalytic zinc, a hinge region, and a hemopexin domain, which provides substrate specificity for many of these enzymes. MMPs are commonly classified on the basis of their substrates and the organization of their structural domains into collagenases, gelatinases, stromelysins, matrilysins, membrane-type (MT)-MMPs, and other MMPs (Apte and Parks, 2015). MMPs are secreted by many cell types including fibroblasts, vascular smooth muscle and leukocytes. MMPs are regulated at the level

of mRNA expression and by activation of their latent (zymogen) form. MMPs are often secreted as an inactive pro-MMP form, which is cleaved to the active form by various proteinases including other MMPs.

While a great deal of effort has been devoted to the study of MMPs and collagen degradation, MMPs are not particularly efficient at degrading fibrillar collagens. Indeed the intracellular lysosomal hydrolases, cathepsins B, L and K, exhibit much higher catalytic rates (k_{cat}) for collagen degradation than do the MMPs (Panwar et al., 2018). Because MMPs exhibit their highest catalytic activity at pH=7.4, MMP-mediated collagen degradation occurs almost exclusively in extracellular environments or on the cell surface (e.g. MT-1 MMP). The initial collagen cleavage event is mediated mainly by the triple helicase collagenases (i.e. MMP1, MMP8, MT-1). However other MMPs, most notably the gelatinases, MMP2 and MMP9, also can initiate degradation of fibrillar collagen, albeit with lower catalytic efficiency.

The degradation of ECM proteins (and particularly collagen) by MMPs, affects not only the structure and mechanical properties of the ECM, but also impacts cell function, proliferation, migration, and adhesion. As MMPs degrade ECM proteins such as collagen and elastin, they can also influence endothelial cell function, Ca^{2+} signaling (Nagano et al., 2004) and ECM contraction.

2. ECM Phagocytosis

The second mechanism by which ECM components (and in particular collagen) are degraded involves intracellular digestion via lysosomal hydrolases. The process of phagocytosis begins with the generation of cell extensions that engulf the collagen fiber and the subsequent formation of caveoli, which are formed at sites where cell adhesion receptors bind to collagen. As a result of membrane-bound proteases (e.g. MT-1), the collagen fiber is cleaved

extracellularly (Lee et al., 2006) and is separated from the adjacent, contiguous fiber. These separated portions of collagen fibers (up to 10 μm in length) are then internalized into phagosomes in which banded (64 nm) collagen fibrils can be observed and where digestion is mediated by cathepsins (Melcher, 1981). The degradation of collagen by phagocytosis is a crucial mechanism for ECM homeostasis (Everts et al., 1996) and is wholly dependent on the formation of cell extensions.

D. Structure of cell extensions involved in ECM remodeling

In collagen phagocytosis, binding of fibroblasts to collagen fibers through activated β1 integrins (Arora et al., 2008) leads to MMP expression and activation , and initiates some of the earliest steps in phagocytosis-mediated ECM remodeling (Arora et al., 2000). Efficient collagen phagocytosis is dependent on extensive remodeling of subcortical actin by proteins like gelsolin (Arora et al., 2013) and adseverin (Tanic et al., 2019), which enable the changes of cell shape that are needed for the generation of cell extensions and for the subsequent internalization of collagen fibers. In fibroblasts involved in collagen phagocytosis, thin membrane extensions have been identified *in vivo* (Melcher, 1981) and *in vitro* correlates of these structures have been defined (Jiang and Grinnell, 2005). Cell extensions in cultured cells exhibit overlapping functions and include lamellipodia, microtubule-based cilia, microtubule-based nanotubes, and actin-based filopodia. Little is known about the role of vimentin in the formation or the function of these structures.

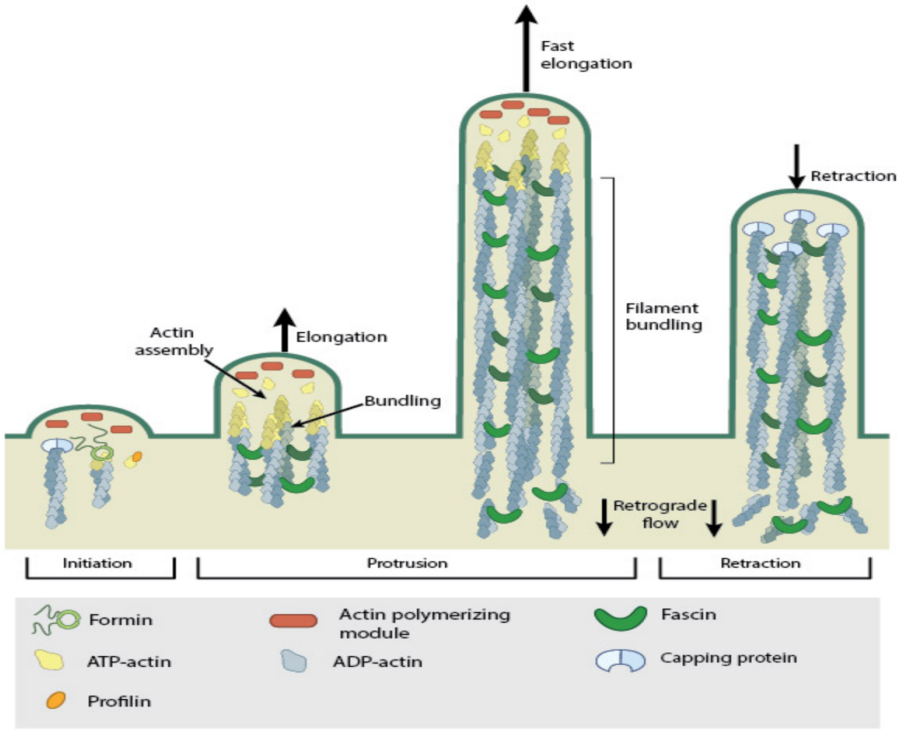


Figure 1: A diagram to illustrate the principal role of actin filaments in the generation of cell extensions and how various actin filament regulating proteins contribute to the formation of these structures.

Of the different types of cell extensions that have been described in cultured cells, lamellipodia and in particular filopodia, are predominant at the leading edges of migrating cells whereas invadopodia and podosomes mainly arise on the ventral surface of the plasma membrane of cultured cells (Alblazi and Siar, 2015). Filopodia probe the ECM, are functionally indispensable in wound healing and contribute to the formation of the long cell extensions that enable collagen phagocytosis and matrix invasion. Filopodia can be further sub-divided into invadopodia, cytonemes, tunneling nanotubes (50-200 µm) and membrane nanotubes (Yamashita et al., 2018). In general, these cell extensions are actin filament-rich structures that contain more than 130 known proteins from different functional protein classes

including WASP, N-WASP, cortactin, the Arp 2/3 complex, Src kinase, and MT-1 MMP. Among the classes of proteins in the filopodia, actin binding proteins are notable as they are enriched at the leading edge of migrating cells and help to promote the formation of cell extensions and later on, to direct migration. Certain actin binding proteins such as cortactin serve as early markers for invadopodia formation and are often overexpressed in various types of invasive cancers (Buday and Downward, 2007).

For collagen phagocytosis and for the tunneling of fibroblasts into the matrix (Willis et al., 2013), the formation of actin-rich cell extensions is of fundamental importance as these cellular protrusions can degrade collagen fibers in the path of the migrating cell. Cell extensions can also wrap around and cleave the collagen fiber; the fragmented fiber is then internalized and enters the phagosomal system for degradation (Rosales and Uribe-Querol, 2017). In the initial steps of cell extension formation, membrane-associated cortical actin at the site of collagen fiber binding is remodeled by actin filament branching proteins including coronins and by actin severing proteins like cofilin and gelsolin (Arora et al., 2000). In concert with actin filament disassembly, actin filament nucleation initiates actin filament assembly, which is also involved in cell extension formation. Later, actin filament depolymerization occurs at the base of the phagocytic cup, which is followed by closure of the phagosome (Freeman and Grinstein, 2014).

Actin-based filopodial extensions are dynamic structures that are generated by the activities of actin nucleators (like formins), actin capping proteins (like Cap Z), actin bundling proteins (like fascins) and I-Bar proteins (Linkner et al., 2014), which integrate cell membrane architecture with sub-cortical actin remodeling (Sudhaharan et al., 2016). Filopodia formation results from continued elongation and convergence of actin filaments in the absence of vasodilator-stimulated protein (VASP) (Bear et al., 2002) or of mDia2 capping of the actin

filament barbed ends (Yang et al., 2007). In addition to actin filaments, tubulin and vimentin intermediate filaments can also be observed in mature cell extensions, where they are required for the elongation (but not for the initial formation) of cell extensions (Schoumacher et al., 2010). For migration into a dense extracellular matrix and to enable collagen internalization in the path of migration, cells must first bind to collagen by integrins. Notably, transport of recycling integrins to the tips of filopodia is critical for early phases of cell attachment to the collagen fiber and for subsequent steps in fiber internalization. In the invasive types of filopodia seen in cancer cells, there is increased expression of the β1 integrin and activation of the small GTPase RhoA; both of these processes correlate with enhanced cancer invasiveness. Formation of invadopodia in cancer cells is also promoted by cdc42, Raf, the formin mDia2, fascin, integrin-linked kinase and β-parvin (Jacquemet et al., 2015).

E. Pericellular proteolysis

ECM degradation involves localized degradation of proteins around the cell (i.e. pericellular proteolysis), which is performed by the tightly regulated activities of a large array of proteases. There are more than 550 known human proteases, which are classified into two broad categories on the basis of their location: membrane-associated proteases and secreted proteases. Of these classes, there are further sub-divisions into 5 distinctive enzyme classes, all of which are controlled by at least 150 endogenous protease inhibitors that are specific to each class. Membrane-type proteases are embedded in the plasma membrane that involve covalent interactions with other membrane proteins whereas secreted proteases are tethered to the plasma membrane through non-covalent interactions with their binding partners such as uPAR. Upon cleavage of the binding domain, the enzymes are then released into the pericellular space. The anchorage of the membrane-associated proteases in particular help to confine their

proteolytic activity to the pericellular space (Rawlings et al., 2014). Membrane-associated proteases degrade a broad array of substrates. For instance, membrane-anchored MMPs (e.g. MT-1) are one of the active enzymes in degradation of pericellular ECM collagen. Other proteases interact with cell surface-associated heparan sulfate proteoglycans, fibrin and fibronectin, which enhance their enzymatic activities by increasing enzyme stability and therefore their catalytic lifetimes.

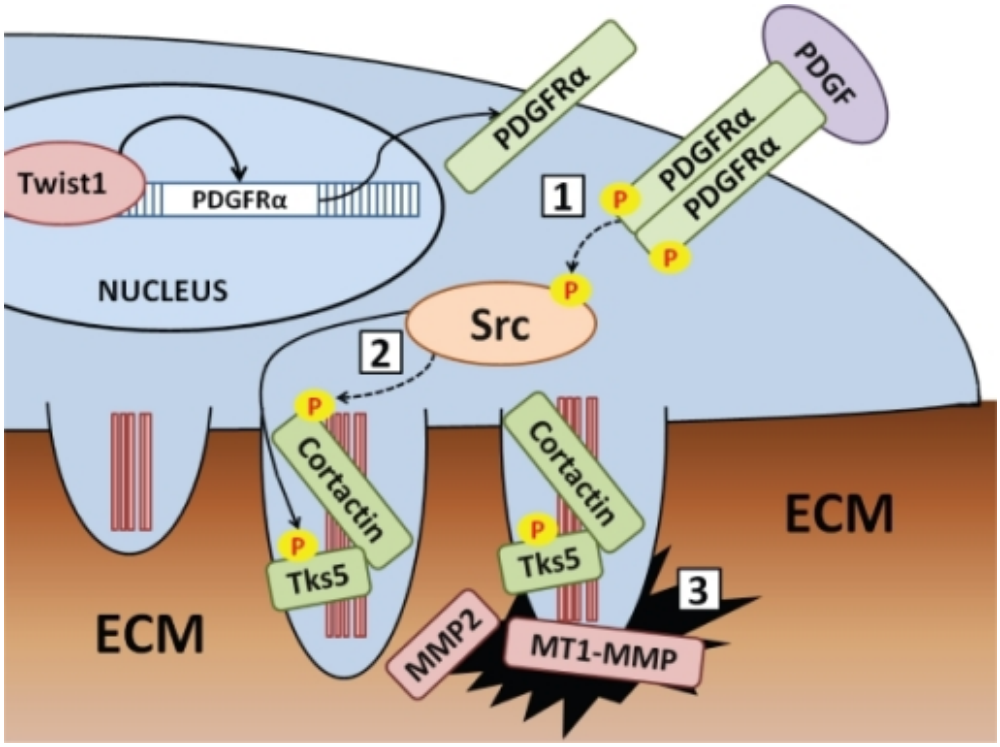


Figure 2: Schematic diagram to illustrate platelet derived growth factor (PDGF)-driven activation of Src, a signaling pathway that leads to the production of cell extensions that are specialized for tunneling into the extracellular matrix (ECM) and which are enriched with matrix-degrading enzymes (e.g. MT1-MMP; MMP2) that facilitate pericellular collagen degradation.

Pericellular proteolysis is dependent on the formation of cell extensions, which are required for cell migration and matrix invasion. The sequestration of proteases in the pericellular space can be mediated by several different types of cell extensions, which include filopodia in normal fibroblasts and invadopodia in cancer cells. Because cell extensions can adhere tightly to the pericellular ECM, they become concentrated sources of proteases that contribute to proteolysis-dependent cell migration. Some of the proteases that are secreted from cell extensions or that are enriched in the invadopodia of cancer cells include MT1-MMP, MMP2, MMP9, ADAM12, ADAM15, ADAM19 (Murphy and Courtneidge, 2011), cathepsins B,L, K (Panwar et al., 2018) and seprase (O'Brien and O'Connor, 2008). Of these proteases, MT1-MMP is especially important for the precise localization of triple helicase activity at discrete membrane binding sites with collagen fibers (Sevenich and Joyce, 2014).

Eukaryotic cells employ either protease-dependent or protease-independent modes for migration and invasion (Wolf and Friedl, 2011). However, without ECM proteases, cells use mechanical forces to physically rearrange the ECM and migrate in an amoeboid-like manner (Lammermann and Sixt, 2009). In protease-dependent cell migration, degradation of ECM molecules is promoted by upregulation or activation of specific enzymes such as MMPs, which are frequently concentrated in the extensions of migrating cells (He and Wirtz, 2014). Among the members of MMP family, MT1-MMP and MMP2 are remarkably abundant in invadopodia, the actin-rich, specialized ECM-degrading membrane protrusions of invasive cells; these findings further implicate the roles of invadopodia in metastatic processes (Poincloux et al., 2009).

F. Involvement of Actin Binding Proteins in Cell Extension Structure and Function

Actin exists in two principal forms: globular (G; or monomeric) actin and polymeric or filamentous (F) actin. From much earlier studies of actin structure, because of the arrowhead pattern that is detectable when myosin decorates actin filaments, the fast-growing end of the polarized filament is by convention designated as the barbed end while the more slowly growing end is named the pointed end. Actin filaments grow in length when ATP-actin monomers are preferentially incorporated into the filament at the barbed end. With filament aging, the ATP that is bound in the central cleft of actin undergoes hydrolysis. The phosphate is released and the ensuing ADP-actin filament disassembles as a result of loss of monomers from the pointed end. These ADP-actin monomers that are released can undergo nucleotide exchange to create ATP-actin monomers, which then participate in a new cycle of actin assembly. This cycle of ATP-hydrolysis-driven, uni-directional filament-growth is named "actin treadmilling".

There are several stages in the *de novo* formation of actin filaments, the first of which is nucleation. Spontaneous actin assembly is not energetically favourable until a nucleus of three interacting monomers are extant. In intact cells, a number of actin binding proteins are essential for rapid nucleation of filaments. Notably, new filaments can form from the side of existing filaments or by severing an existing filament to create a new actin barbed end. There are a large array of different actin binding proteins that fulfill these different nucleating functions (Winder and Ayscough, 2005)**.** One of the most well-known nucleating systems for actin filament assembly involves the Arp2/3 complex, which nucleate filaments from the sides of existing filaments (Pollard and Borisy, 2003). Actin polymerization is also nucleated by

formin proteins that processively add actin monomers to the barbed end of filaments (Xu et al., 2004).

A large group of actin binding proteins regulate actin filament growth, stability and disassembly and involve such high abundance actin capping and severing proteins as gelsolin (Burtnick et al., 2004). Other groups of actin binding proteins bind to the sides of actin filaments (α-actinin; VASP) and regulate the structure of actin arrays and various signaling systems. They also regulate higher order actin filament structures and the formation of actin bundles (e.g. fimbrin in microvilli). Finally, actin filaments are frequently organized into orthogonal arrays, which is mediated by proteins or protein complexes that contain multiple actin-binding domains. At the leading edge of migrating cells and in the phagocytic cup adjacent to collagen fibrils undergoing phagocytosis, actin filament cross-linking proteins like filamin A or spectrin contain two domains separated by longer, more flexible spacer regions, which enables more orthogonal arrangements of actin filaments. Because of earlier work that linked filamin A to the spreading of cultured cells (Kim et al., 2008), the formation of cell adhesions and the integration of vimentin filaments in the spreading process (Lynch et al., 2013), I focussed the first part of my research programme on potential interactions between filamin A and vimentin that may impact the formation of cell extensions.

G. Filamin A Structure and Function

Filamins are a family of actin binding proteins that organize and crosslink actin networks into dynamic, three-dimensional structures. These proteins are particularly abundant at cell membranes where they integrate cell adhesion with multiple signaling pathways. Three isoforms of filamin (A, B, C) have been identified. Filamin C is restricted to muscle cells while filamin A (FLNA) or ABP-280, is the most abundant member of the family and its expression

is broadly distributed across many types of cells. FLNA can promote high angle branching of actin filaments, which stabilizes plasma membranes that otherwise would be susceptible to shear force-mediated distortion or rupture (Kim and McCulloch, 2011). FLNA has a dimeric structure comprised of two monomers, each of which contains an actin binding domain and a rod segment, which consists of 24 highly homologous repeats of about 96 amino acid residues each. Two intervening calpain-sensitive hinge regions (H1 and H2) are inserted in between repeats 15 and 16, and repeats 23 and 24 respectively (Fucini et al., 1999).

FLNA plays essential roles in the mechanical stabilization of cells and in mechanically-induced signaling pathways in particular (Shifrin et al., 2009). Because of its versatile functions, it is perhaps not surprising that FLNA interacts with a broad array of other proteins. Indeed, FLNA binds more than 90 known proteins including ion channels, receptors, intracellular signaling molecules, and transcription factors. Many of these proteins are kinases (e.g. p21 activated kinase, ROCK, AKT, PKC) that are critical in cell signaling pathways that regulate cell adhesion through integrins and responses to mechanical forces (Nakamura et al., 2011).

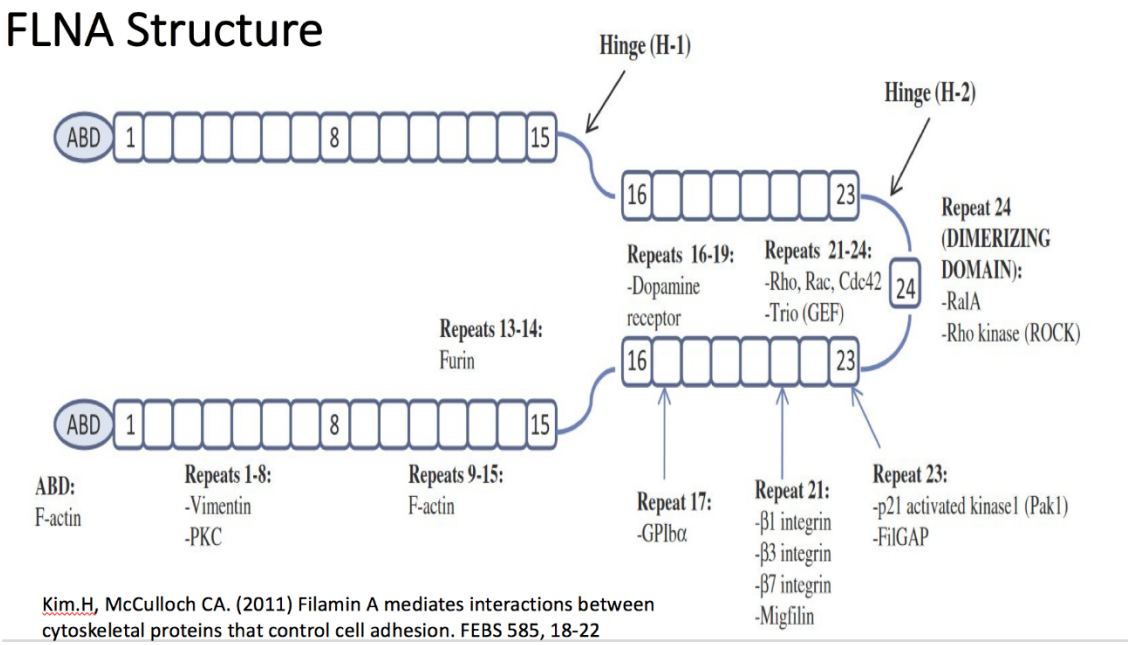


Figure 3. Diagram of filamin A structure (from Kim and McCulloch, 2011; FEBS Lett) showing the domains of filamin A and some of the interacting proteins.

The interactions of FLNA with various binding partners are modified by mechanical forces, phosphorylation, proteolysis, competitive binding and/or multimerization of the protein binding partners (Nakamura et al., 2011). The mechanical deformation of the C-terminal of FLNA could, for example, alter interactions between neighboring repeat pairs with force levels that are sufficient to expose the cryptic integrin binding site (Lad et al., 2007; Pentikainen and Ylanne, 2009). Conversely, phosphorylation of FLNA, especially at Ser2152, is believed to regulate integrin binding most likely by facilitating force-induced dissociation of the auto inhibition that constitutively decreases the force requirement (Chen et al., 2009).

In addition to its interactions with actin-associated proteins, FLNA may also regulate transcription as a result of its translocation to the nucleus. In this process the FLNA hinge region is cleaved by calpain, which generates 2 rod sequences and a self-association domain

(Gorlin et al., 1990). Translocation of the C-terminal fragments of FLNA to the nucleus is followed by series of interactions with specific transcription factors, which result in an inhibition of cell spreading (but not migration) by altering the dynamics of the expression of focal adhesion proteins at the transcriptional level (Heuze et al., 2008; Kwak et al., 1993). Notably, regulation of focal adhesion turnover and function, which is important for cell adhesion and migration, is also impacted by competitive binding of other integrin binding proteins such as talin, kindlins, ICAP1,14-3-3, CD98, Shc, and β3 endonexin (Legate and Fassler, 2009). On the other hand, increased FLNA-integrin binding activity can be realized through integrin clustering at focal adhesion sites (Carman and Springer, 2003).

H. Roles of FLNA in cell attachment to ECM and remodeling of ECM

FLNA anchors multiple transmembrane proteins to the actin cytoskeleton, thereby providing a scaffold for a wide range of signaling pathways. One of the most important ways by which FLNA participates in the remodeling of ECM is through β1 integrin signaling. Integrin activation through application of tensile forces through ECM proteins recruits FLNA to actin filaments in collagen-bound integrins, which also facilitates the spreading of cells to the matrix (D'Addario et al., 2001). FLNA also actively regulates ECM degradation. For example, knockdown of FLNA increases the expression of MMP9, which induces activation of MMP and then promotes pericellular matrix protein degradation (Baldassarre et al., 2012).

FLNA is typically distributed throughout the cytoplasm of cells although FLNA molecules that interact with β1 and β7 integrins are more abundant at the leading edges of migrating or cytokine-activated cells (Nakamura et al., 2014). The generation of cell extensions that attach to collagen requires a concerted and well-integrated network of activated β1 integrins, small GTPases, and actin-binding proteins. FLNA facilitates recruitment of vinculin

and paxillin to focal adhesions, and regulates β1 integrin and small GTPase activation (Kim et al., 2010b). These processes are crucial for the formation of cell extensions seen in migrating cells and is facilitated by ligand binding via inside-out integrin signaling (Kim et al., 2008). Inhibition of the ability of FLNA to interact with certain binding proteins compromises cell motility and the detachment of cancer cells from primary tumor sites (Jiang et al., 2013). Because of its critical role in promoting cell migration, FLNA has become a promising molecular target for development of new drugs to block the spread of metastatic cancers.

I. Intermediate Filaments

Intermediate filaments (IFs) are comprised of a large family of proteins that form cytoplasmic, insoluble filaments, ~10 nm in diameter, which are intermediate in size between thick filaments (myosin) and microfilaments (actin). Based on their amino acid sequences, several distinctive classes of IF proteins can be identified in different cell types and tissues. Class I and II IFs are the keratins expressed by epithelial cells. Class III IFs include the proteins vimentin in mesenchymal cells, desmin in myogenic cells, peripherin and glial fibrillary acidic protein in glial cells. Class IV IFs comprise the neurofilament proteins L, M, H, and internexin. Class V IFs comprises the nuclear lamins; Class VI Ifs include the protein tanabin (Steinert and Spath, 1984).

IF networks are highly dynamic and exhibit several different levels of complexity. For example, more than one type of IF subunit can co-exist in a single cell: keratin and vimentin IFs can form separate networks in the same cell, based on evidence showing that colchicine, collapses only vimentin networks but not keratin, suggesting that these two IF systems are differentially susceptible to an agent that is usually associated with disruption of the

microtubule protein, tubulin (Aynardi et al., 1984). In contrast, desmin and vimentin are both susceptible to colchicine and can copolymerize into the same filament (Steinert, 1981).

J. Structure of Vimentin

All IFs contain conserved regions of large, central coiled-coil α-helical rod domains of similar size, which extend into four tracts of α-helices that are flanked by two non-α-helical end domains. In contrast, the N-terminal and C-terminal domains of IFs vary widely in size and sequence. All IFs are composed of sub-filamentous or proto-filamentous particles, which individually consist of a three-chain, coiled coil unit (Steinert et al., 1980), or a pair of two-chain coiled coils. These structural predictions were originally based on analyses of sequence and cross-linking experiments (Gruen and Woods, 1983). Additional studies using transmission electron microscopy indicate that there are two mass forms of IFs: "light-weight" IFs with 22-23 subunits/50 nm of filament length and the "mature" IF with 33-34 subunits/50 nm of filament length (Steven et al., 1983). Variations in the structural properties and functions of different classes of IFs are thought to arise from variations in the protruding terminal sequence of the subunits that are located at the periphery of IFs (Steinert et al., 1983).

K. Mechanisms of vimentin filament assembly

Vimentin filament assembly is a dynamic and complex process that has not been studied in the same depth as say, actin filament assembly. In brief, the general assembly mechanism can be divided into three phases. In phase 1, there is lateral association of tetramers into unit-length filaments (ULFs). In phase 2, longitudinal annealing of ULFs into protofilaments occurs. In phase 3, mature filaments of+ ~10 nm in diameter are assembled (Herrmann and Aebi, 1998). Arising from these notions of vimentin assembly, ULFs are the basic building blocks of the

assembly process and their formation is also highly dynamic. ULFs are released from growing filaments at very low rates. The equilibrium between free ULFs and assembled filaments favours polymerization because IF proteins are polyelectrolytes with an acidic rod domain and a basic head domain that leads to the formation of a complex and insoluble network of filaments that is driven by ionic interactions and hydrophobic interactions. Consequently, mature IFs cannot be easily dissolved with non-ionic detergents and salts, even at high concentrations (Herrmann and Aebi, 2016). Nevertheless, as seen for vimentin, IFs are very responsive to post-translational modifications (e.g. protein kinase-mediated phosphorylation), which can favor vimentin disassembly/assembly processes (Inagaki et al., 1987). For measuring vimentin assembly in cultured cells, an index of vimentin assembly can be estimated in which the ratio of detergent-soluble vimentin to insoluble vimentin is quantified (Li et al., 2006).

Vimentin phosphorylation plays a crucial role in regulating vimentin disassembly and spatial reorganization during mitosis or in response to extracellular stimuli (Goto et al., 1998). Potential phosphorylation sites in vimentin include, but are not limited to phosphorylation of Ser 6, 33, 38 or 39, 55 or 56, 71, 72, and 82. Some of the candidate kinases that mediate vimentin phosphorylation include, but not are limited to PKA, PKC, ROCK, AKT, PAK, Cdk1, CAMKII, Aurora B, Plk1 and MAPKAP-K2 (Cheng et al., 2003; Ivaska et al., 2005; Yamaguchi et al., 2005; Yasui et al., 2001). Because of the dynamic nature of vimentin filament assembly and the lack of detailed knowledge of the regulatory mechanisms, how phosphorylation drives assembly is not well understood.

L. Function of Vimentin in Mesenchymal cells

The increased expression of vimentin is one of the most prominent markers for epithelial-mesenchymal transition (EMT), a process that is important in development, wound healing, and in the metastasis of cancer cells (Dongre and Weinberg, 2019). EMT initially is manifest in rapidly migratory cell types such as when embryonic ectodermal cells migrate into the mesodermal cleft. In these early precursors of mesenchymal cells, keratin genes are inhibited and expression of vimentin is strongly increased (Franke et al., 1982). Postnatally, vimentin expression is observed in fibroblasts, endothelial cells, lymphocytes, specialized brain cells, and tumour cells (Franke and Moll, 1987). The increased expression of vimentin in cells and EMT conversion is thought to be important in enhancing the migratory potential of epithelial cells to the mesenchymal type of cell migration (Dongre and Weinberg, 2019).

Cell migration is very much dependent on the tightly regulated functions of actin networks, which are organized by different actin binding partners into well-ordered arrays and which support the formation of membrane protrusions such as lamellipodia and filopodia (Gardel et al., 2010). Vimentin filaments may interact with actin filaments directly via their tail domain (Esue et al., 2006), and indirectly via cytolinker proteins like plectin (Svitkina et al., 1996) but the pathophysiological impact of these interactions is not defined. Transverse arcs, which are actin bundles containing non-muscle myosin II, exhibit retrograde flow of small vimentin particles ("squiggles"), which may contribute to vimentin filament turnover (Prahlad et al., 1998) and that may mediate transportation of squiggles to the perinuclear membrane. In the absence of vimentin expression, transverse arcs retract from the leading edge, indicating a potential role of vimentin in controlling actin dynamics (Jiu et al., 2015). Notably, increased vimentin expression is associated with enhanced cell motility, loss of intercellular contacts,

and increased turnover of focal adhesions (Gabbiani et al., 1982), suggesting a role for vimentin in affecting the remodeling of the ECM.

Cell adhesion and migration appear to depend on the well-orchestrated functions of actin, microtubule and intermediate filament networks. Nevertheless, the functional relationships and the critical mechanisms by which these networks interact remain elusive. Based on tentative earlier data, it seems that FLNA and vimentin may interact to control cell migration, a notion that is supported by co-localization of these proteins in mesenchymal cells and tumor cell extensions (reviewed in (Kim and McCulloch, 2011). Further, there is a marked decrease in the abundance of vimentin in Src-transformed cells that are surrounded by FLNA knockdown cells (Kajita et al., 2014), suggesting a potential signaling system in which FLNA affects vimentin expression.

II. Statement of the Problem

The actin, microtubule and intermediate filament cytoskeletons are critical functional determinants of myriad cellular processes. These cytoskeletal networks are intimately involved in the function of several signaling pathways, contribute to developmental, tissue homeostatic and pathological systems and help to maintain cellular integrity in the face of environmental stressors. The actin cytoskeleton has been examined in detail as it strongly impacts the control of cell growth and migration, which are crucial for embryogenesis, wound healing and the invasion of healthy tissues by cancer cells. One of the important proteins that is involved in the regulation and function of the actin cytoskeleton is the actin binding and cross-linking protein filamin A (FLNA), which contributes to the formation of orthogonal arrays of sub-cortical actin filaments, regulates integrin activation and promotes lamellipodia growth and the formation of cytoplasmic protrusions. Complete deletion of FLNA in mice is embryonic-lethal (Feng et al., 2006). FLNA knockdown in cultured fibroblasts gives rise to abnormal phenotypes in which cells exhibit retracted lamellipodia and weaker attachment to extracellular matrix proteins.

In addition to the actin cytoskeleton, intermediate filaments (IFs) are major contributors to cell growth and movement and, for cells of mesenchymal origin, vimentin (VIM) is the most abundant IF protein. In addition to its fundamental role in the support of cell structure, vimentin may interact with actin filaments through its C-terminal tail and possibly through various cross-linking proteins. Currently the mechanisms by which the actin and vimentin cytoskeletal systems functionally interact are not well-defined. Deletion of vimentin results in impaired cell adhesion and migration, a phenotype which is also exhibited by FLNA knockdown cells. In preliminary experiments I found that vimentin IF assembly is enhanced by FLNA, suggesting a potential functional interaction between these two proteins. Currently, the molecular

mechanisms and regulatory processes by which FLNA affects vimentin filament assembly are not known.

III. Global Hypothesis

Filamin A binding proteins enable the assembly of vimentin filaments, which contribute to the formation of cytoplasmic extensions and are involved in early steps in cell migration and matrix invasion.

IV. Specific Hypothesis

Filamin A functionally contributes to the structure of the intermediate filament cytoskeleton by tethering kinases that phosphorylate vimentin and thereby enable vimentin filament assembly. As a result of these processes, filamin A and vimentin collectively contribute to the formation of cytoplasmic extensions, structures that are critical for early steps in cell invasion and matrix remodeling.

V. Objectives

1) Assess phenotypic changes in fibroblasts that are associated with the knockdown of FLNA and vimentin.
2) Examine vimentin filament distribution in FLNA WT and KD fibroblasts.
3) Identify potential molecules that interact with FLNA that may affect vimentin filament assembly.
4) Assess whether FLNA-associated kinases affect the phosphorylation and assembly of vimentin filaments.
5) Evaluate FLNA and vimentin-associated mechanisms that contribute to cell extension formation.

VI. Materials and Methods

Reagents

Rabbit monoclonal anti-filamin A [EP2405Y] and anti-vimentin (phospho-vimentin S72; EP1070Y) antibodies were purchased from Abcam (Cambridge, MA). Anti-PAK1 antibodies were obtained from Cell Signaling Technology (Danvers, MA). Chicken polyclonal anti-vimentin antibody was purchased from Novus Biologicals (Littleton, CO). Fibronectin, IPA-3 (a specific PAK 1 inhibitor), withaferin A, 3,3'-Iminodipropionitrile (IDPN) and puromycin were obtained from Sigma-Aldrich (St. Louis, MO). Alexa Fluor® 488 goat anti-mouse, Alexa Fluor® 568 goat anti-rabbit antibodies, and rhodamine phalloidin, were from Life Technologies (Burlington, ON). Alexa Fluor® 647 AffiniPure F $(ab')_2$ fragment donkey anti-chicken IgY (H+L) was from Jackson ImmunoResearch (West Grove, PA). Y-27632 was from Calbiochem (Millipore; Billerica, MA).

Cell Culture and Transfection

NIH-3T3 fibroblasts that constitutively express FLNa (FLNa WT) or cells transfected with FLNa short hairpin RNA (FLNa KD) were obtained from David Calderwood (Yale University). Cells were cultured at 37°C in complete DMEM containing 10% Hyclone fetal bovine serum and 1% antibiotics). FLNa KD cells required a selective growth medium supplemented with 1.5 μg/mL puromycin to maintain the efficacy of the FLNA knockdown. For vimentin and PAK1 siRNA transfection, cells were trypsinized, plated on 100 mm dishes, and immediately transfected with Dharmacon ON-TARGETplus transfection reagents (Lafayette, CO) for 48 hr as described by the manufacturer. Cells were trypsinized, washed, collected and re-plated on FN (1 mg/ml) for 3 h in medium containing 1% serum before stimulating with 20 ng/ml IL-1 or vehicle control in serum-free medium for various time points. Whole cell lysates were collected and protein concentrations were determined by BCA assay. Equal amounts of total

proteins from each treatment condition were separated on 10% acrylamide gels and immunoblotted to estimate the effectiveness of the knockdown.

Immunoblotting and immunoprecipitation

For immunoblotting cells were lysed on ice, sedimented at 12,000 rpm for 4 minutes and protein concentrations were determined by the BCA protein assay (Pierce). Equal amounts of proteins were loaded on individual lanes of 8% SDS-PAGE gels, separated by electrophoresis and transferred to nitrocellulose membranes. The membranes were blocked with 5% BSA-TBS and probed with the indicated antibody followed by goat anti-mouse, goat-anti rabbit, or donkey-anti chicken fluorophore conjugated secondary antibodies as appropriate. All immunoblots were performed in triplicate. Blot density was quantified with a Li-Cor Odyssey imager (Lincoln, NB). The ratios of blot densities of the protein of interest to loading protein controls were analyzed. In some experiments, the relative proportions of vimentin in the pellets and supernatants of fractionated cell lysates was computed from blot densities to estimate the Vimentin Assembly Index.

For immunoprecipitation, cells were lysed in 1% Tris-NaCl-Triton immunoprecipitation buffer (20 mM Tris at pH 7.5, 1% Triton X-100, 0.1% SDS, 150 mM NaCl) containing 1 mM PMSF, 1 mM NaVO3, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Equal amounts of protein from cleared extracts were immunoprecipitated with the Dynabeads immunoprecipitation protocol (Invitrogen, Carlsbad, CA, USA) using the primary antibodies as indicated.

Mass Spectrometry

Filamin A was immunoprecipitated as described above and proteins were eluted from immunoprecipitation beads with 50 mM glycine buffer (pH 2.3–2.5). The eluted proteins were dialyzed for 36 h in carbonate buffer (25 mM NH4HCO3 at pH 7.5). Trypsin (1 μg; Roche

Diagnostics, Rotkreuz, Switzerland) was added to the sample, which was rotated overnight at 37°C. Subsequently, 0.1% acetic acid was added to the sample, which was then air-dried with an evaporator. Lyophilized samples were analyzed by TMT-MS on a QStar XL Hybrid LC/MS/MS System (Applied Biosystems, Foster City, CA, USA; MDS Sciex, Concord, ON, Canada) at the Hospital for Sick Children SPARC BioCentre (Toronto, ON, Canada). Scaffold 4.0 (Proteome Software, Portland, OR, USA) was used for analyzing search results, calculating P values for each peptide match and matching peptide spectra.

Immunostaining

Cells were plated on fibronectin-coated glass-bottom MatTek 8-chamber slides (~10,000-15,000 cells per well), incubated at 37°C, 5% CO_2 for specific time points. Following incubation, samples were washed with 1x PBS, fixed with 4% paraformaldehyde, permeabilized with 0.2 % Triton X-100, stained with DAPI in NP-40 (10 µg/ml) and rhodamine phalloidin for actin filaments. For protein expression studies of FLNa, vimentin, PAK1, ROCK, phospho-vimentin s72, s39, and s56, samples were incubated with appropriate primary antibodies (in 0.2% BSA-PBS) for 1 hour at 37°C. Cells were washed with 1x PBS following primary antibody incubation. Corresponding secondary antibody incubation and PBS wash were performed in the same manner for 1 hour. After fixation with 4% paraformaldehyde for 15 min, cells were permeabilized with 0.2% Triton X-100, blocked for 1 h in 0.2% BSA, and stained with either the appropriate antibodies or fluorescent affinity dyes. A TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with a ×40 oil-immersion objective lens) was used to determine the spatial distribution of proteins of interest.

Transmission Electron Microscopy

cell monolayers on collagen were fixed in 4% paraformaldehyde and 0.6% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for 1 h at RT and washed with three changes of the same buffer for 45 min. Cells were immunostained for vimentin as described above and then 10 nm diameter nano-gold particles coupled to rabbit-anti-chicken antibody was used to detect bound anti-vimentin antibody. Cells were post-fixed in 1% osmium tetroxide (Marivac Canada, Saint-Laurent, Quebec) in phosphate buffer for 45 min and washed with two changes of the buffer for 30 min. Cells were dehydrated through a graded series of ethanol to absolute ethanol (25, 50, 70, 95, and 100% ethanol). A mixture (2:1) of Epon 815 and Araldite (Marivac Canada) was used to infiltrate the specimen through a graded series of the resin mixture diluted in ethanol (30, 50, and 60% Epon-Araldite) over 3 h and then in 100% Epon 815-Araldite overnight. Next day, fresh 100% Epon 815-Araldite was added to specimens and polymerized for 48 h at 60°C. To cut cross-sections of the monolayer, the blocks were detached from the tissue culture plastic, re-embedded over a thin layer of 100% Epon-Araldite, and polymerized overnight. Light-gold sections (70 nm in thickness) were cut with a diamond knife and mounted on copper grids. The sections were stained with uranyl acetate and lead citrate (Fisher Scientific, Pittsburgh, PA) and examined under a scanning transmission electron microscope (H-7000; Hitachi, Tokyo, Japan) operated at 75 kEV. Section analysis was performed on vimentin immunostained cell samples to determine the lengths of stained vimentin filaments. Prior to microscopy analysis of samples, the cell lysate preparations were stained with chicken antibodies to mouse vimentin followed by secondary antibody (rabbit anti-chicken antibody) coupled to 25 nm diameter immunogold (Electron Microscopy Services).

Transwell Cell Extension Assay

For assessment of the formation of cell extensions through fibronectin-coated pores, FLNa WT and KD cells were cultured on T-75 flasks (Becton Dickinson, Oakville, ON) in complete

growth medium and grown to sub-confluence. Cells were starved for 3 hours before being seeded on to six-well polycarbonate membrane Transwell® inserts (8.0 μm pore size; 10μm thickness; Corning, Tewksbury, MA). Prior to the seeding, inserts and bottom chambers were coated with fibronectin for 30 min at 37°C. For establishing a chemotactic gradient, chemoattractant (20% FBS, DMEM) was placed in the bottom chamber and cells were seeded with serum-free growth medium in top chamber on the inserts. Samples were incubated at 37°C for 6 hours and then fixed and immunostained with appropriate primary antibodies and analyzed by confocal microscope as described above.

Fractionation Assay

Cells grown to 70% - 80% confluency in 150 mm cell culture dish were placed on ice. After removal of the culture medium, cold phosphate buffered saline (PBS; 5 ml) was used to wash the cells twice. Next, 5 mL of ice-cold cell lysis buffer (50 mM PIPES, 50 mM NaCl, 5% Glycerol, 0.1% NP-40, 0.1% Triton X-100 and 0.1% Tween 20) was added to the dish and kept on ice for 1.5 min. Lysates were collected and kept on ice for further use. Cytoskeletal proteins remaining bound to the dish were then rinsed using 5 ml of cold Tris-HCl buffer three times on ice, and solubilized/denatured in 500 μL of 1% SDS. The total protein concentration was determined using the BCA protein assay (Pierce). All the buffers used during the cytoskeleton extraction procedure contained protease (Roche protease inhibitor cocktail) and phosphatase inhibitors (5 mM NaF, 2 mM sodium vanadate and 10 mM β-glycerolphosphate).

In vitro Kinase Assay

For removal of phosphate groups from serine, threonine and tyrosine residues, recombinant VIM (100 mg/ml) was incubated with alkaline phosphatase in a buffer (10 mM Tris-HCl pH 8.5, 5 mM $MgCl_2$, 0.2 mM $ZnCl_2$; 800 U alkaline phosphatase from bovine intestinal mucosa) for 30 min, 37°C with agitation. The reaction was terminated by the addition of 100 mM

imidazole and 2 mM Na_2VO_3 (final concentration). Vimentin phosphorylation was performed in kinase buffer (25 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 0.1 mM Na_2VO_3, 5 mM b-glycerolphosphate, 2 mM DTT) in the presence or absence of PAK1 (100 ng/ml) and IPA3 (5 mM). The reaction was initiated by the addition of 0.4 mM ATP-$MgCl_2$ for 30 min at room temperature and the reaction was stopped with 4x Laemmli sample buffer. Samples were boiled for 10 min and load in equal amounts on 10% SDS-PAGE. Proteins were transferred to nitrocellulose membrane and incubated overnight at 4°C with a-phospho-VIM (S56 or S72) and vimentin primary antibodies. Membranes were washed, incubated with secondary antibodies, visualized and analyzed as described above.

Statistical Analysis

All experiments were conducted in quadruplicate and were repeated ≥3 times. For continuous variables, means ± standard errors of the mean were computed. A Student's t test was used for 2-sample comparisons, and statistical significance was set at a type I error rate of $p<0.05$. For multiple comparisons, ANOVA and post hoc Tukey's test were used.

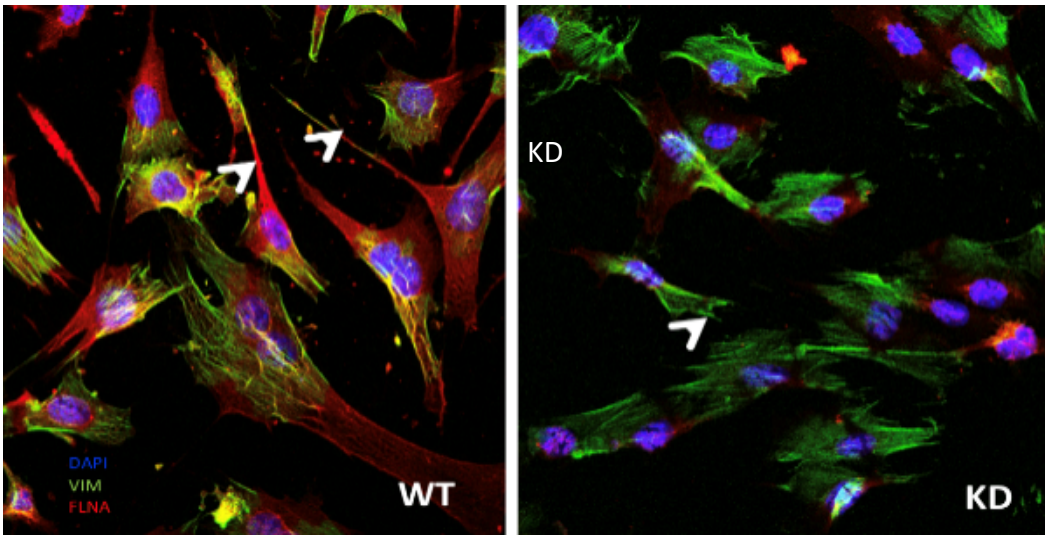
VII. Results

Knockdown of FLNA reduces cell extension formation

As a first step in examining the functional relationship between FLNA and vimentin in the generation of cell extensions, I measured cell extensions in a previously described cell line that is stably transfected with shRNA against mouse FLNA (FLNA KD, (Shifrin et al., 2009). FLNA WT or KD cells were plated on fibronectin-coated glass for 6 hours, fixed with paraformaldehyde, immunostained for FLNA and vimentin, counter-stained with DAPI (for nuclei), and the numbers and lengths of cell extensions were quantified as described (Yuda et al., 2018). The number of cell extensions in FLNA KD cells was reduced by 4-fold compared with FLNA WT (****$p<0.0001$; $n>120$ cells for each cell type; **Fig. 1B**). In FLNA KD cells, immunostained vimentin intermediate filaments disappeared from the tips of the cell extensions. The length of cell extensions was 1.5-fold shorter in the FLNA KD cells compared with the FLNA WT cells (*$p<0.05$), suggesting that FLNA is involved in the initiation of cell extension formation and the growth of individual extensions once this process is initiated. Analysis of cell migration ability through 8 μm pore size in the 3D-transwell membrane showed faster migration in FLNA WT than KD cells (Fig. 1C Left). WT cell populations that successfully crossed the 10 μm thick membrane, reached the bottom surface and reinitiated spreading. FLNA KD cells exhibited slower migration across the membrane and few cells were able to spread on the bottom surface of the membranes (Fig. 1C Right). Further, transmission microscopy analysis of FLNA WT and FLNA KD cells (immunolabelled with nanogold reagents) demonstrated sparse protofilaments in KD samples as compared to the WT, in which protofilaments were gathered to form bundle-like structures (Fig. 1D).

Figure 1

A


DAPI
VIM
FLNA
WT
KD
KD

B

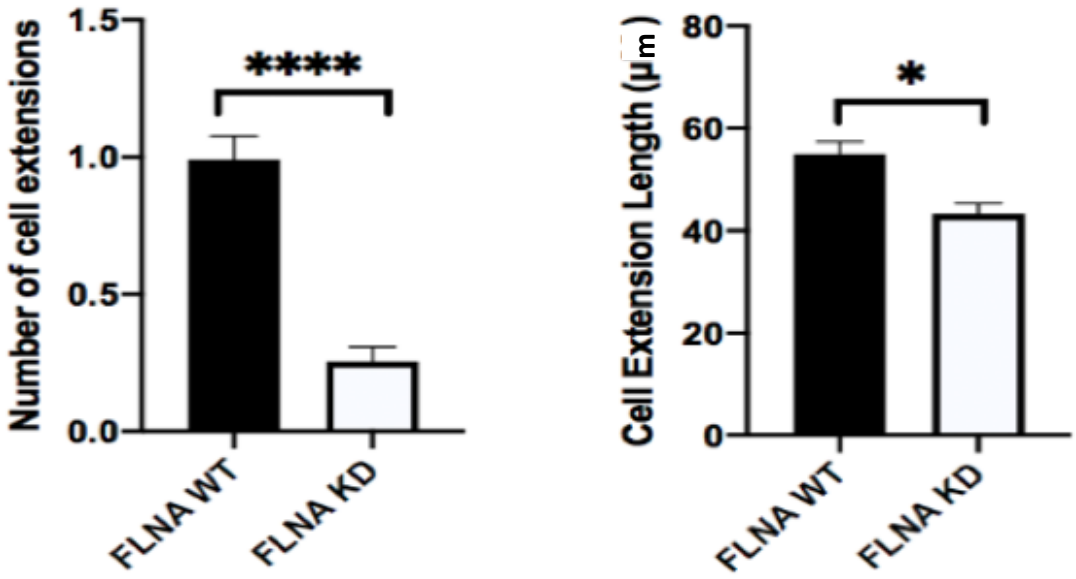

Number of cell extensions
1.5
1.0
0.5
0.0
****
FLNA WT
FLNA KD
Cell Extension Length (µm)
80
60
40
20
0
*
FLNA WT
FLNA KD

Figure 1C

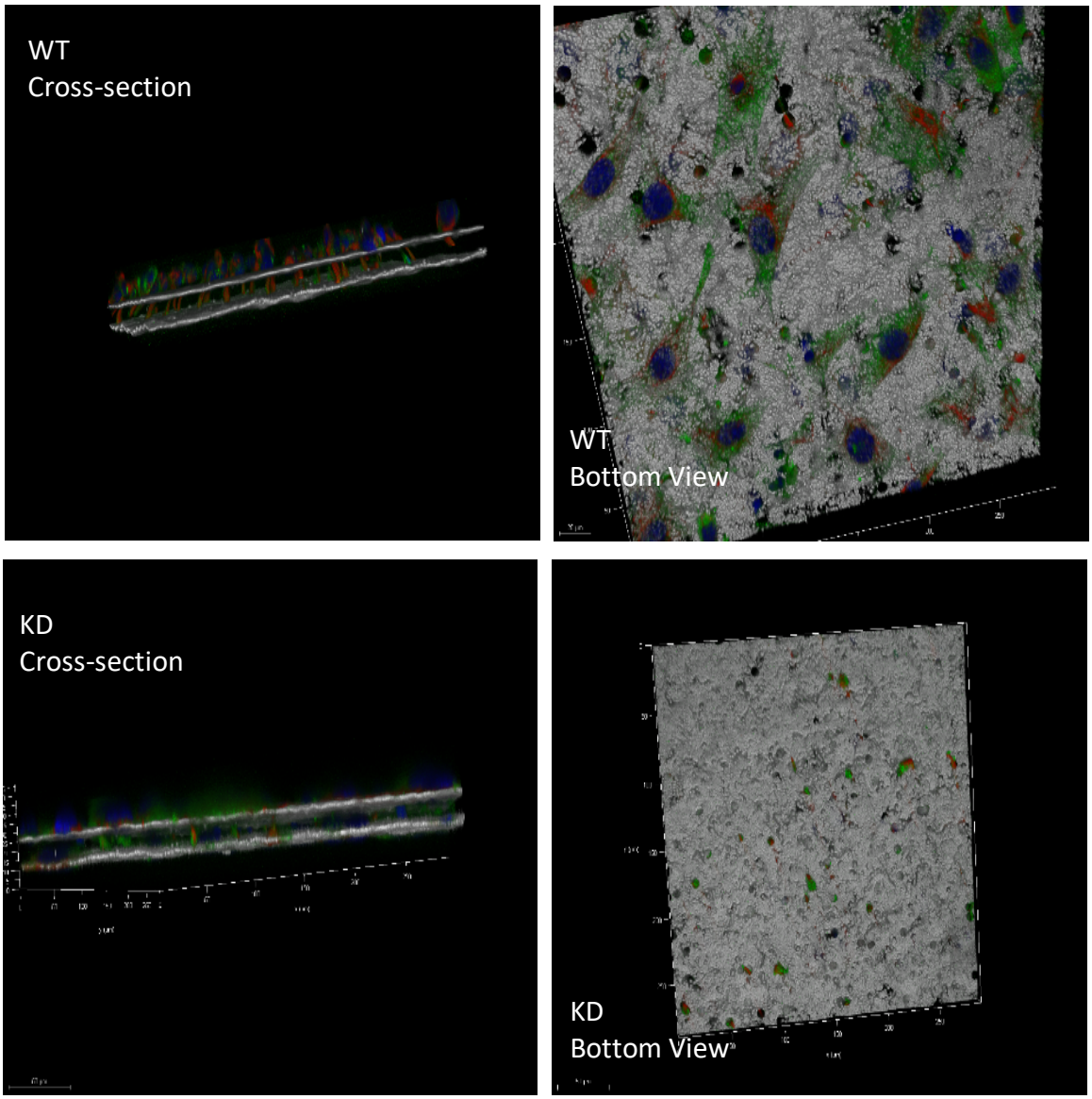

WT
Cross-section
WT
Bottom View
KD
Cross-section
KD
Bottom View

Figure 1D

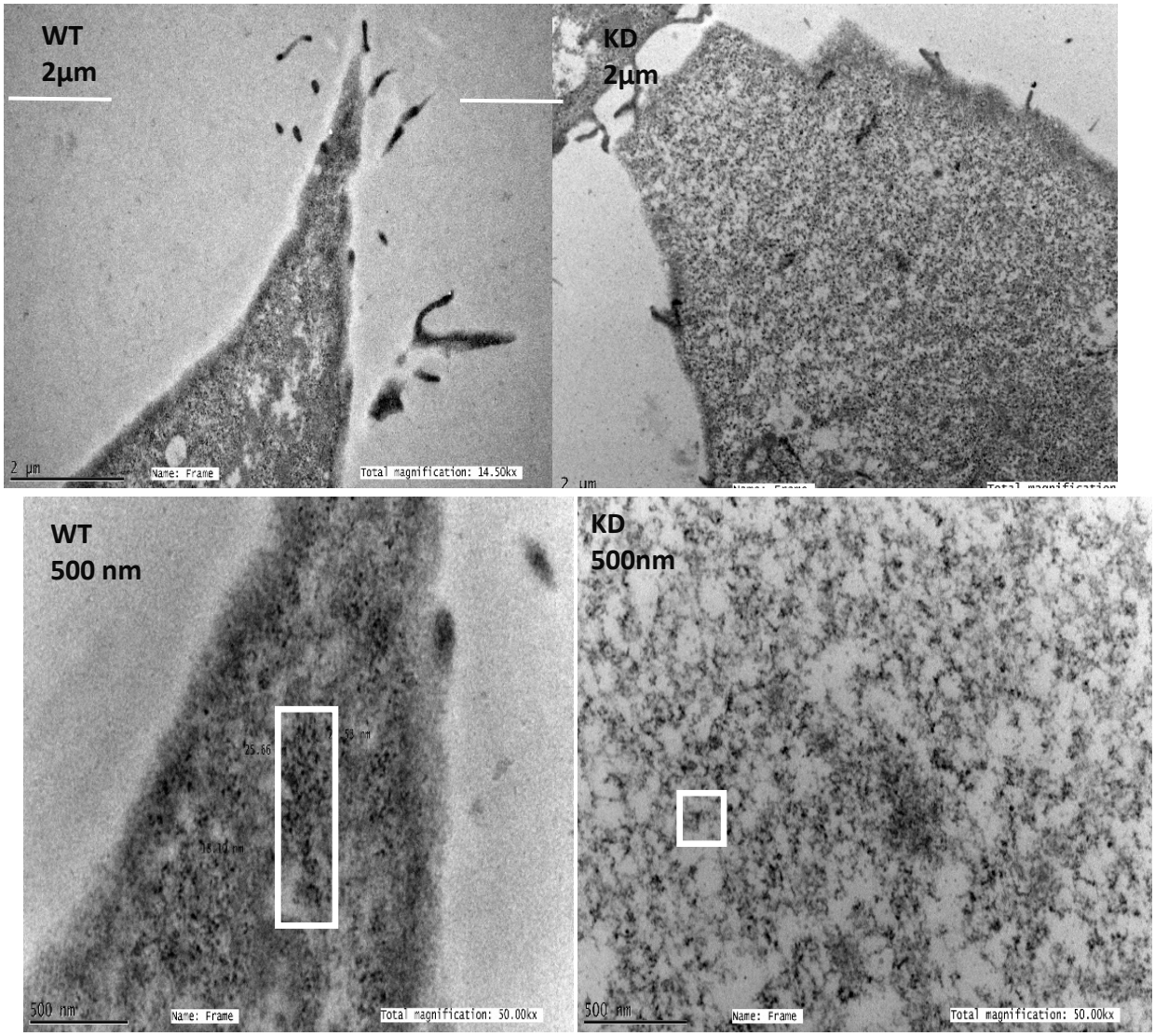

WT
2µm
2 µm
Name: Frame
Total magnification: 14.50kx
KD
2µm
2 µm
WT
500 nm
500 nm
Name: Frame
Total magnification: 50.00kx
KD
500nm
500 nm
Name: Frame
Total magnification: 50.00kx

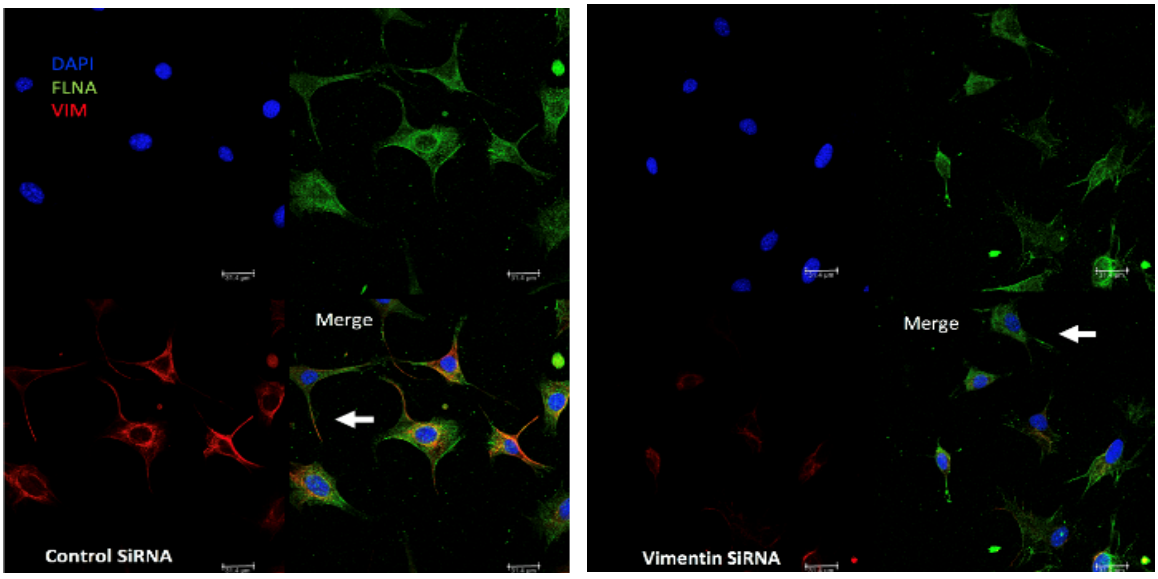
Fig. 1 (A) FLNA WT and KD cells were fixed, permeabilized, and immunostained for vimentin filaments (in green; secondary antibody- Alexa-Fluor 488 conjugated goat anti-rabbit) and for FLNA (in red; secondary antibody- Alexa-Fluor 647 conjugated donkey anti-chicken antibody). Cell nuclei were stained with DAPI. White arrows point to cell extensions. **(B)** Quantification of the number of cell extensions per cell and length of extensions was performed with Image J ($n > 120$ cells for each cell type). Data are mean ± s.e.m. *$p < 0.05$ KD, and ****$p<0.0001$ compared with WT cells. **(C)** 3D Transwell assay showed faster migration in FLNA WT cells as many of which successfully crossed the 10μM thick membrane, reached the bottom surface, and reinitiated spreading event. However, FLNA KD cells were mostly still in process of migration after 6hr incubation, none of which were able to establish spreading event at the bottom surface. (**D**) FLNA WT and KD cells were immunolabeled with vimentin antibody followed by second antibody conjugated to gold nanoparticles (25 nm) and imaged using transmission electron microscopy. At high magnification, there were immunolabeled, bundles of vimentin filaments in FLNA WT cells, whereas in FLN KD cells, immunolabeled vimentin was present as “squiggles” with no evidence of mature filament assembly

Transient knockdown of vimentin decreases the length of cell extensions

As knockdown of FLNA inhibited cell extension formation, I examined whether vimentin expression levels also affect cell extension formation. Wild type 3T3 cells were transfected with siRNA for mouse vimentin, re-plated on fibronectin-coated surfaces for 6 hr, and immunostained as describe above. Loss of vimentin caused a slight alteration in cellular morphology **(Fig. 2A),** but did not affect the average number of cell extensions **(Fig. 2B right)**. The length of extensions in vimentin-deficient cells was 8-fold shorter than WT cells (**p<0.001) **(Fig. 2B left),** suggesting that vimentin contributes to the processes that promote the growth of extensions but not to the initiation of extension formation.

Figure 2

A.



B.

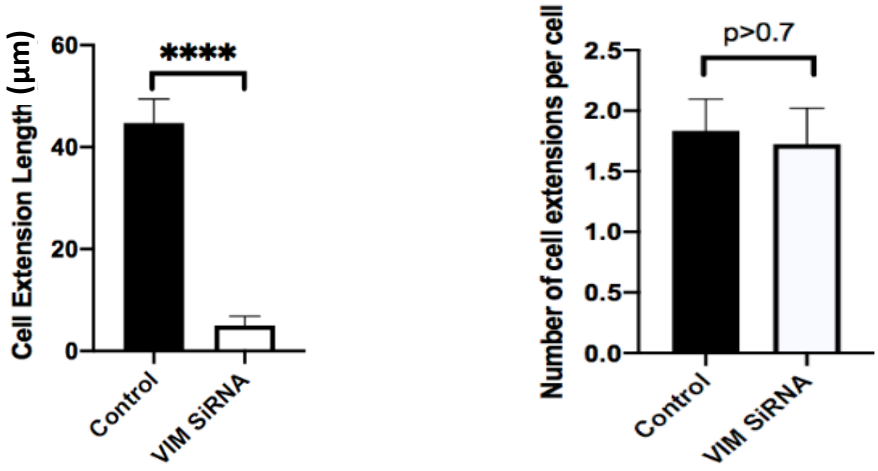


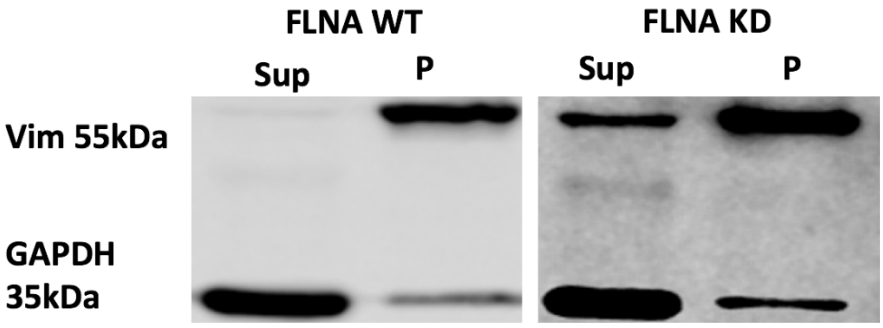
Fig. 2 (A) Vimentin knockdown-treated FLNA WT cells were fixed, permeabilized and immunostained for FLNA (in green; secondary antibody- Alexa-Fluor 488 conjugated goat anti-rabbit) and for vimentin (in red; secondary antibody- Alexa-Fluor 647 conjugated donkey anti-chicken antibody). Cell nuclei were stained with DAPI. **(B)** Quantification of cell extension length and numbers was performed with ImageJ (n>30 cells per group). Data are mean±s.e.m. ****p<0.0001. Comparison of irrelevant siRNA control cells (siRNA-) with siRNA knockdown (siRNA+) in the mean number of cell extensions.

Vimentin filament assembly is affected by FLNA expression

Based on detergent solubility, there are two principal forms of vimentin in cells, the relatively smaller molecular mass (22-23 subunits/50 nm of filament length) soluble filaments and the larger molecular mass (33-34 subunits/50 nm of filament length) insoluble mature filaments (Steven et al., 1983). These vimentin filament populations can be separated by fractionation in 1% Triton at 10,000 *g*, in which the protofilaments are concentrated in the supernatants whereas the more mature filaments are largely found in the cytoskeletal pellet fraction. In cells expressing FLNA, there was little soluble vimentin (Fig. 3A). However, when FLNA was knocked down, there was a large increase in the abundance of soluble vimentin filaments, which evidently were not converted into the mature insoluble form. There was a 6-fold increase of the vimentin filament disassembly index (ratio of soluble/insoluble vimentin) in cells with FLNA knockdown (Fig. 3B), indicating that FLNA may affect the maturation of vimentin filaments.

Fig. 3

A.



B

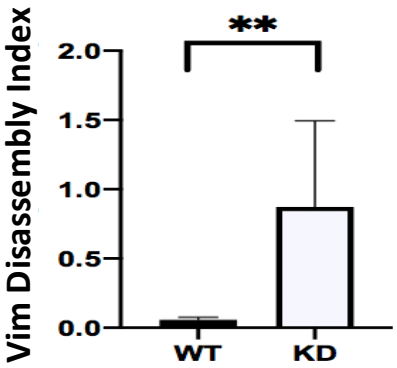
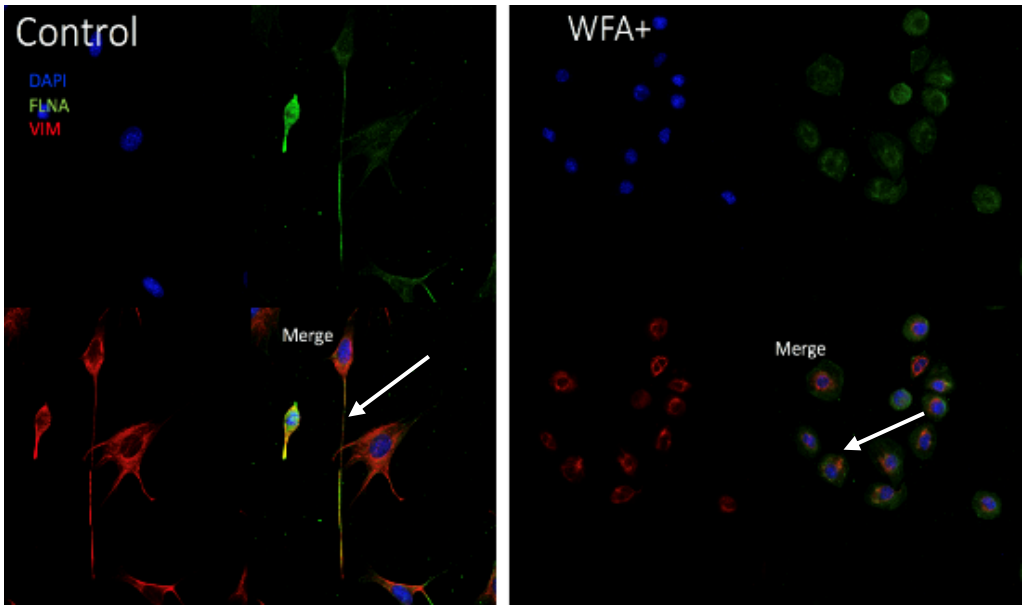


Fig. 3. FLNA WT and KD cells were plated on fibronectin for 4 hours, lysed, fractionated into supernatants and pellets, loaded on to 8% SDS-PAGE gels and analyzed by immunoblot. **(A)** Densitometric analysis shows higher abundance of soluble vimentin in cells with knockdown of FLNA. **(B)** Computation of vimentin disassembly index (density of soluble vimentin divided by the insoluble) shows 6-fold higher vimentin disassembly index in FLNA KD cells (Data are mean±s.e.m. **$p<0.01$).

Inhibition of vimentin assembly blocks cell extension formation

I investigated the role of vimentin filament assembly in cell extension formation. FLNA WT cells were treated for 6 hr with withaferin A (WFA, 5 μM), a naturally occurring anti-tumor and anti-angiogenic agent that induces collapse of vimentin filaments (Mohan and Bargagna-Mohan, 2016). Cells were plated, fixed, immunostained, and imaged as described above. There was complete block of cell extension formation (Fig. 4A), indicating that inhibition of vimentin filament assembly affects cell extension formation. Similar results (Fig. 4B) were observed when FLNA WT cells were treated with 2% iminodipropionitrile (IDPN), a selective inhibitor of vimentin assembly (Kumar et al., 2007). Both IDPN and withaferin (Ridge et al., 2016) affect the assembly of vimentin filaments from precursors but are reported to not affect the abundance of vimentin protein in cells.

Figure 4



B.

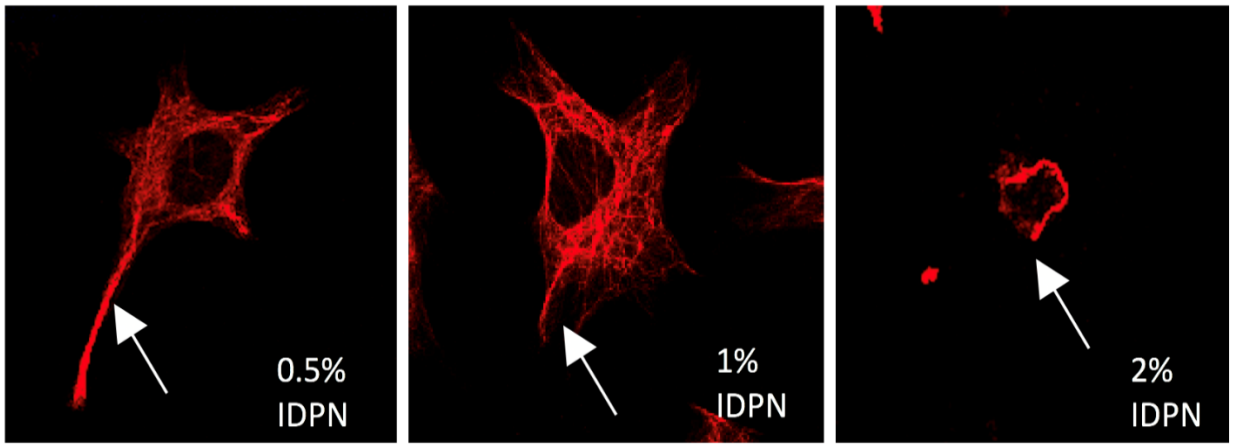


Fig. 4 Prior to treatments, FLNA WT cells were plated on fibronectin-coated substrates and incubated at 37°C, a crucial step to initiate cell extension formation. **(A)** After plating and initial attachment of cells to the fibronectin-coated surfaces, cells were treated with WFA (5 μM) and incubated for 5 hr at 37°C. Cells were immunostained for FLNA with Alexa-Fluor 488-conjugated goat anti-rabbit (green), and for vimentin with Alexa-Fluor 647-conjugated donkey anti-chicken antibodies (red). Confocal microscopy imaging showed extensive retraction of cell extensions that accompanied the collapse of vimentin filaments. **(B)** A similar protocol was used for IDPN dose- response experiments (0.5%, 1%, or 2% IDPN) and these results showed marked retraction of cell extensions with 1% IDPN, and complete retraction with 2% IDPN.

Effect of WFA and IDPN on vimentin sedimentation

As treatment with WFA or IDPN blocked cell extension formation (Fig. 3), I considered that loss of these structures is linked to inhibition of vimentin filament assembly. Analysis of the relative abundance of soluble and mature vimentin filaments showed that WFA and IDPN increased the abundance of the soluble vimentin pool (Fig. 5A), which was quantified by estimation of the vimentin disassembly index (Fig. 5B).

Fig. 5

A **B**

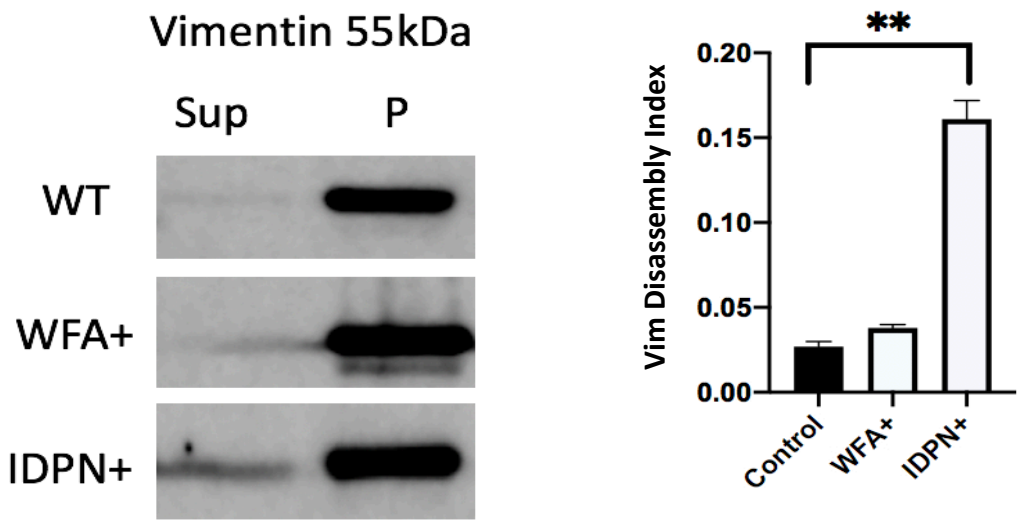


Fig. 5 After plating on fibronectin, followed by treatment with WFA (5 μM) or 2% IDPN for 6 hr, FLNA WT cells were lysed, fractionated into supernatant and pellets and loaded on to 8% SDS-PAGE gels for immunoblot analysis. **(A)** Densitometry of immunoblots show that WFA and more so, IDPN, reduced the abundance of insoluble vimentin filaments and increased the abundance of vimentin protofilaments in the soluble fraction. **(B)** Computation of vimentin disassembly index shows no difference in WFA treated samples but a 4-fold increase (IDPN) in vimentin disassembly.

Relationship of vimentin filament assembly and phosphorylation

Conversion of soluble vimentin protofilaments to insoluble mature filaments is strongly affected by vimentin phosphorylation (Eriksson et al., 2004). Examination of vimentin phosphorylation in FLNA WT and KD cells showed that in the absence of FLNA, there was marked reduction of vimentin serine 72 phosphorylation (Fig. 6A), suggesting that FLNA-interacting kinases may affect vimentin phosphorylation. Accordingly, I screened for enzymes that would affect vimentin filament assembly by tandem mass spectroscopy. I focussed in particular on kinases that demonstrate marked differences of abundance in the soluble and insoluble fractions as vimentin phosphorylation by kinases is thought to be important for regulation of vimentin assembly (Chou et al., 1996; Eriksson et al., 2004). I considered that there may be obvious differences in the abundance of potential, vimentin-phosphorylating kinases in the soluble fraction compared with the insoluble fraction since I anticipated that the kinases would be more abundant in the fraction in which vimentin was phosphorylated (and therefore with more abundant filaments). Two of the kinases that demonstrated differences in protein abundance in the insoluble fraction versus the soluble fraction were Prp4 and PKCα (Fig. 6B), however, neither of these proteins showed consistent differences of abundance in the soluble and the insoluble fractions by western blot analysis (data not shown).

Previous data have shown that several FLNA-binding kinases may contribute to vimentin phosphorylation; these include PKC, PI3K, ROCK, and PAK1 (Snider and Omary, 2014) (Kim and McCulloch, 2011; Kim et al., 2010b). To identify enzymes that phosphorylate vimentin and that affect filament maturation and cell extension formation, I treated cells with selective kinase inhibitors that were specific for each candidate enzyme and cell extension formation was then measured. Protein kinase C (PKC), which binds to FLNA (Kim et al., 2010a) and is thought to be an important mediator of vimentin phosphorylation (Ivaska et al.,

2005) was evaluated by treatment of FLNA WT cells with bis(indolyl)maleimide (BIM; IC 50=10 nm; for in vitro kinase assays; 1000-fold higher for intact cells), a specific PKC inhibitor, for 6 hours (at 2.5 μM, 5 μM, or 10 μM). Cell extension lengths were measured after the treatments. Analysis of these data indicated no statistically significant differences ($p>0.2$) between the treated and control groups, or among the treatment groups treated with different concentration of BIM (Fig. 6C). These data indicated that PKC does not affect vimentin-dependent cell extension formation.

A similar experimental approach was used to test the potential role of PI3K using LY294002, a PI3K inhibitor. FLNA WT cells were incubated with LY294002 at 1.5 μM, 3 μM, 6 μM, 12 μM, 24 μM, 48 μM, and 96 μM. In these experiments, cell extension length was reduced only at high concentrations of LY294002, which could be toxic to cells (Fig. 6D). Accordingly, PI3K was not considered to be a good candidate that would affect cell extension formation. When ROCK activity was inhibited with Y-27632, a ROCK-specific inhibitor (Shah and Savjani, 2016), surprisingly, the length of cell extensions was increased ($p<0.01$) in KD cells (Fig. 6E), suggesting a potential role for ROCK in pathways that control cell extensions, but not in actively promoting the elongation of cell extensions.

Role of p21 activated kinase (PAK1) in vimentin filament assembly

I examined a potential role for PAK1 using IPA-3, a PAK1 selective inhibitor (IC50=2.5 μM). Of those cells remaining adherent to the substrate, there was complete blockade of cell extension formation (5 μM IPA-3; Fig. 6F) and cells also exhibited collapsed vimentin filament structures compared with untreated cells. As PAK1 appeared to be a potential candidate kinase for phosphorylation of vimentin, I conducted a cell fractionation assay and found a 7-fold increase ($p<0.003$) in vimentin disassembly index after IPA-3 treatment (Fig. 6G). Taken

together these data suggest that PAK1 plays a central role in vimentin-dependent cell extension formation.

Figure 6A

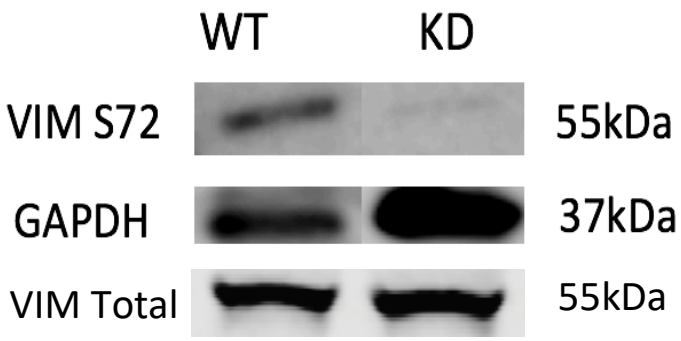


Figure 6B

:							WT S Reference		WT P Treatment 1		KD S Treatment 2		KD P Treatment 3	
Identified Proteins [71/1587]	Accessic	Alterna	Molecu	Protein	null	Logâ„ F	Taxono	Sample	Std Dev	Sample	Std Dev	Sample	Std Dev	Sample	Std De
Creatine kinase B-type OS=Homo	P12277	CKB	43 kDa	TRUE			unknow	0.002	1.001	0.023	0.989	1.254	1.167	0.608	1.23
Serine/threonine-protein kinase P	Q13523	PRPF4B	117 kDa				unknow	-0.008	0.943	1.541	1.02	0.072	0.93	0.755	0.917
Epidermal growth factor receptor	Q12929	EPS8	92 kDa				unknow	-0.002	1.053	-0.046	0.977	-1.1	0.934	-1.892	0.945
Phosphoinositide 3-kinase regulat	Q9Y57(	PIK3R4	153 kDa	TRUE			unknow	0.003	0.927	0.022	0.927	1.6	0.927	1.187	0.927
Adenylate kinase isoenzyme 1 OS:	P00568	AK1	22 kDa				unknow	0.003	1.002	0.283	0.917	1.359	0.905	0.622	0.918
Protein kinase C alpha type OS=H	P17252	PRKCA	77 kDa	TRUE			unknow	0.003	0.943	-1.824	0.943	-1.23	0.943	-3.066	0.943
Eukaryotic elongation factor 2 kin	O00418	EEF2K	82 kDa	TRUE			unknow	0.003	0.921	-1.073	0.921	-0.142	0.921	-0.945	0.921
Tyrosine-protein kinase BTK OS=H	Q06187	BTK	76 kDa				unknow	0.003	0.909	1.5	0.909	0.616	0.909	1.31	0.909
Cyclin-dependent kinase 9 OS=Ho	P50750	CDK9	43 kDa	TRUE			unknow	0.003	0.89	-1.267	0.89	-0.061	0.89	-1.248	0.89

Figure 6

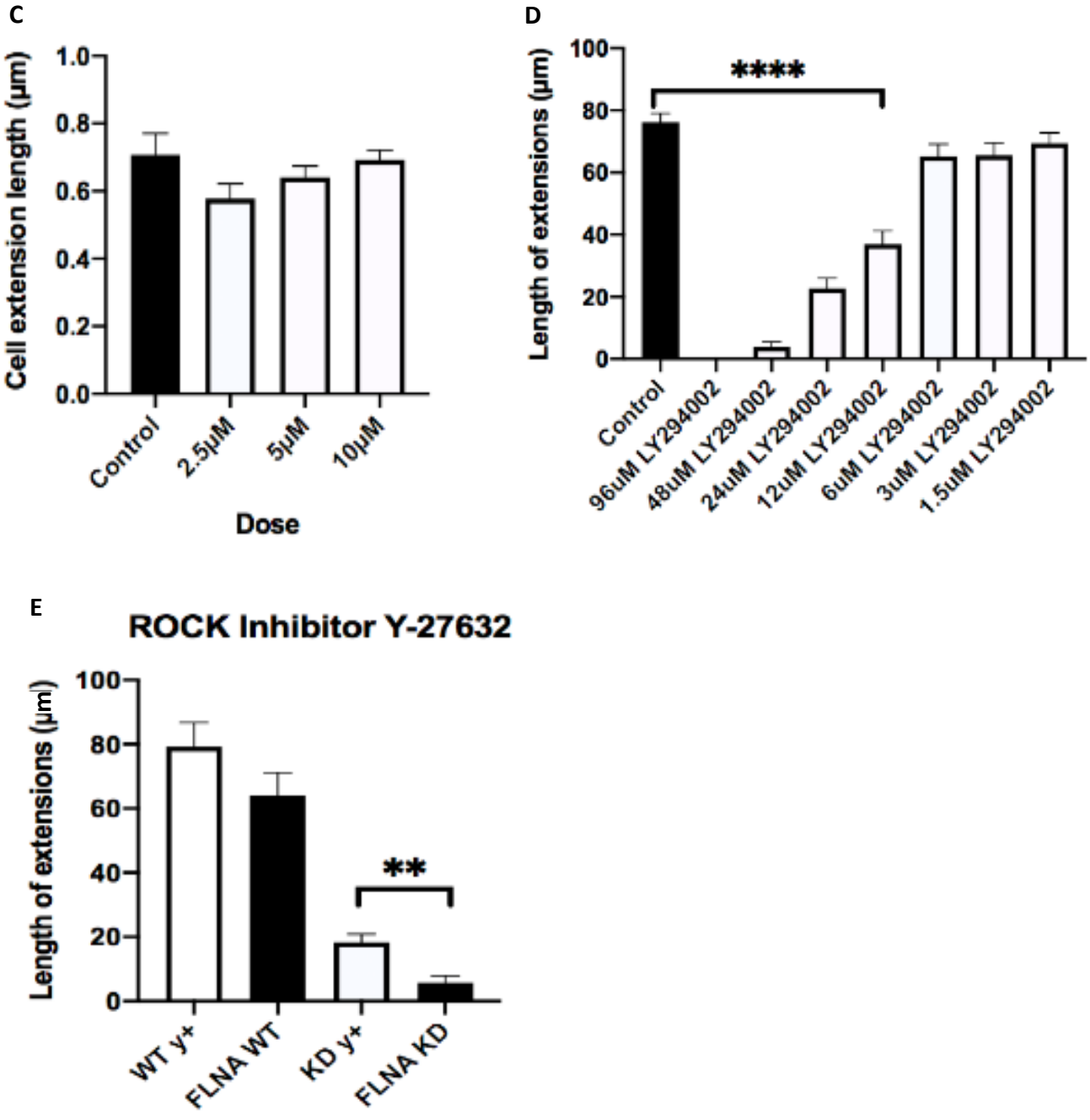

C
Cell extension length (µm)
1.0
0.8
0.6
0.4
0.2
0.0
Control
2.5µM
5µM
10µM
Dose
D
Length of extensions (µm)
100
80
60
40
20
0
****
Control
96uM LY294002
48uM LY294002
24uM LY294002
12uM LY294002
6uM LY294002
3uM LY294002
1.5uM LY294002
E
ROCK Inhibitor Y-27632
Length of extensions (µm)
100
80
60
40
20
0
**
WT y+
FLNA WT
KD y+
FLNA KD

Figure 6F

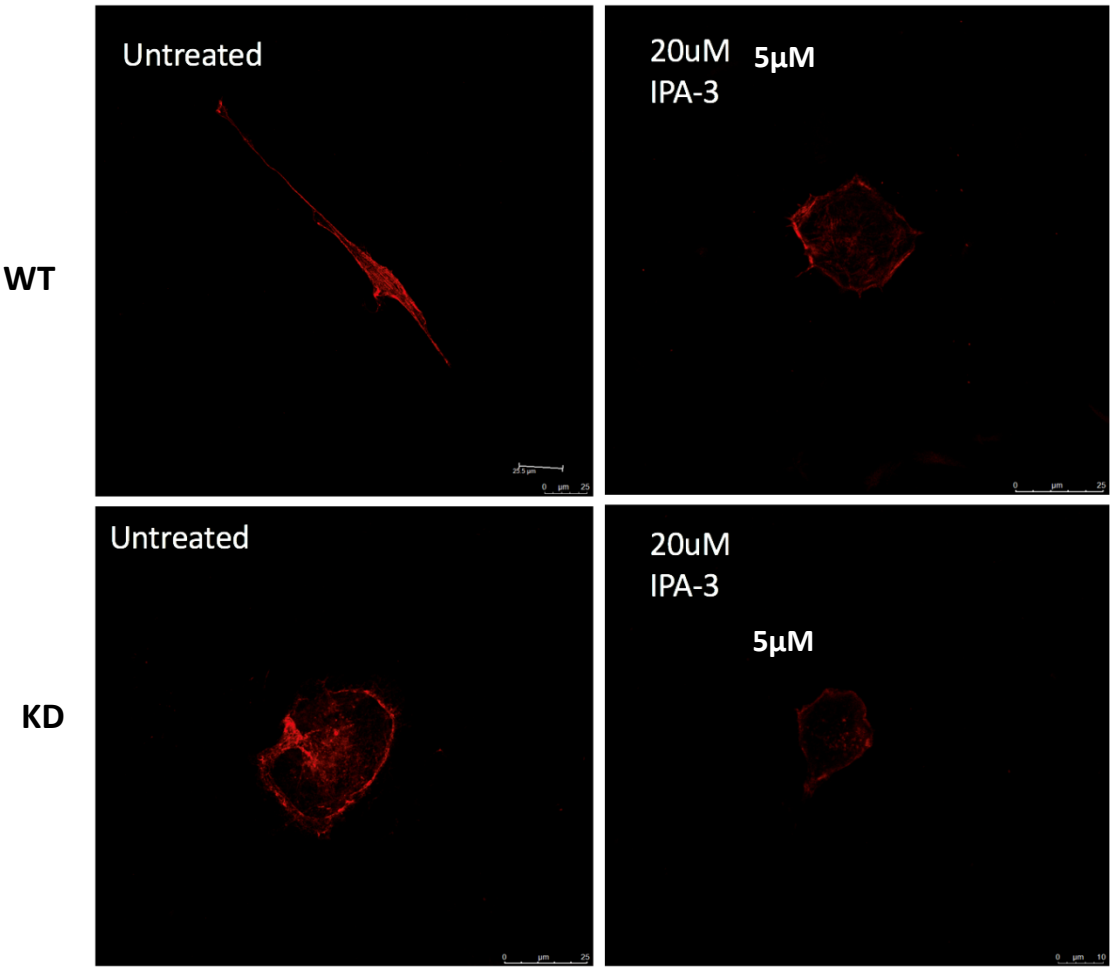


Figure 6G

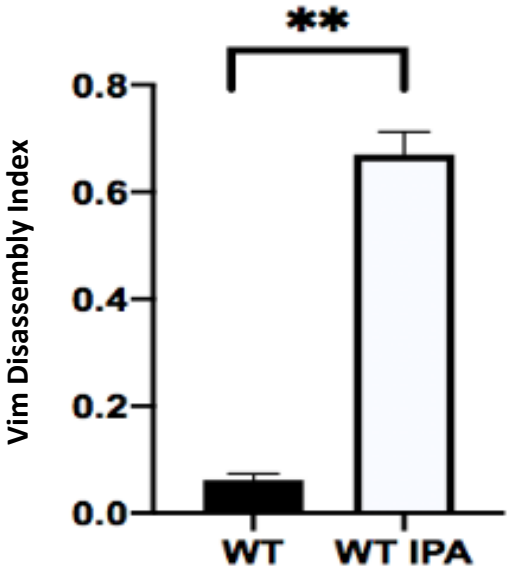


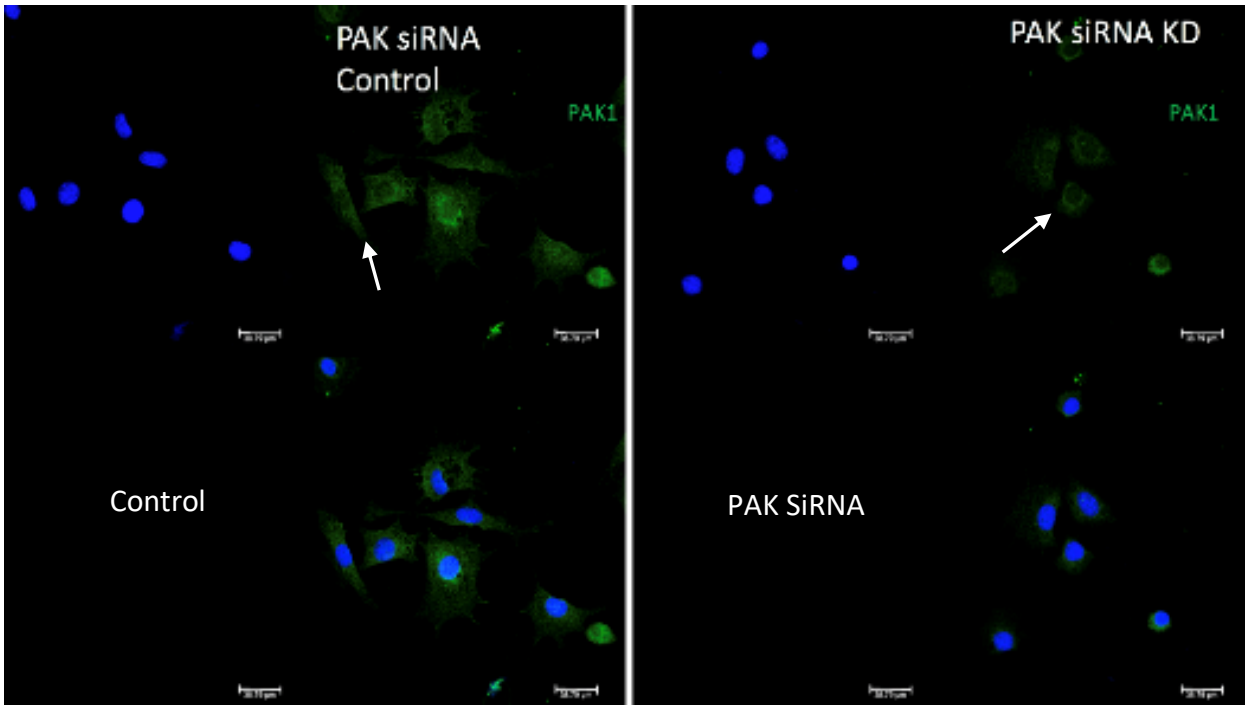
Fig. 6 Role of kinases in cell extension formation. (**A**) Phosphorylated vimentin (serine 72) was assessed in FLNA WT and KD cells by Western blot analysis. Protein loading was standardized with GAPDH. There was higher vimentin phospho-serine 72 in FLNA WT compared with KD cells (F). (**B**). Tandem mass spectrometry analysis of fractionated lysates (pellets and supernatants; prepared as described in the Materials and Methods) of FLNA WT and FLN KD cells. The data are the fold-enrichment or fold-reduced (log to the base 10) of the number of high probability ($p<0.001$) peptides that were enriched (green-highlighted) or reduced (brown highlighted) in the respective samples. Cells were allowed to spread for 3 hours on fibronectin prior to preparation for mass spectrometry. (**C**). FLNA WT cells were plated on fibronectin-coated surfaces, allowed to spread for 1hr, and treated with BIM at 2.5, 5 or 10 μM. Cell extension lengths were assessed as described in the Materials and Methods section. Data are reported as mean±s.e.m. There were no statistically significant differences ($p>0.2$) in cell extension length before and after BIM treatment. (**D**) A PI3K inhibitor (LY294002) dose-response assay was performed using the same experimental design. There were reductions of extension formation but only at high doses of LY294002 (treatment concentration > 6μM), which could be attributable to a cytotoxic effect on the cells. (**E**) The effect of the ROCK inhibitor, Y-27632, on cell extension formation was examined in FLNA WT and KD cells. This inhibitor did not affect the formation of cell extensions in WT cells ($p<0.7$) and reduced cell extension length in KD cells (**$p<0.02$). (**F**) The PAK inhibitor IPA-3 was tested (at 5 μM IPA-3). Stained cells showed strong inhibition of cell extension formation in FLNA WT cells. (**G**) The effect of IPA-3 on vimentin filament assembly was assessed by fractionation and western blot analysis. Computation of the vimentin assembly index indicated a >10-fold increase after IPA-3 treatment (**$p<0.007$).

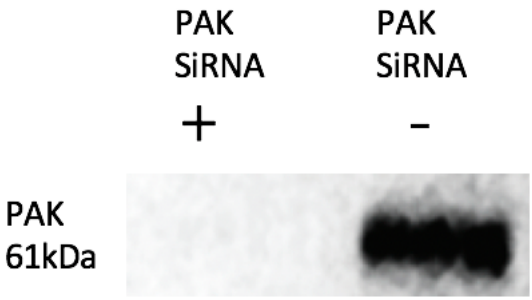
Deletion of PAK1 inhibits formation of cell extensions and blocks phosphorylation (Ser 72)

Inhibitors reduce kinase activity but may exert off-target effects. Accordingly, I used siRNA knockdown as an alternative approach to assess the importance of PAK1. Following knockdown of PAK1, FLNA WT cells were immunolabeled for PAK1 (**Fig. 1A top**) and actin (**Fig. 1B**), immunoblotted for PAK1 (**Fig. 1A bottom**) to assess knockdown the efficiency and the impact of PAK knockdown on cell extension formation. With PAK1 knockdown, ~80% of cells exhibited loss of PAK1 in immunostaining and immunoblotting (**Fig. 7A**). For cells that showed loss of PAK1 staining, there was a substantial reduction in the mean cell extension length as measured in cells counterstained for actin filaments (**Fig. 7B, C**). Notably, fractionation of cell lysates and western blot analysis after PAK1 knockdown showed marked vimentin filament disassembly (**Fig. 7D**, ~2-fold difference, $**p<0.02$). These data indicated that PAK is important for promoting vimentin assembly from soluble protofilaments to insoluble mature filaments. Further, when I immunoblotted phospho-vimentin S72 after PAK1 knockdown, there was a large reduction of phospho-vimentin S72 in PAK KD samples compared to the WT (**Fig. 7E**), which is consistent with the notion that PAK1 phosphorylates vimentin serine72 and thereby promotes vimentin filament maturation. Vimentin serine72 expression was also checked in FLNA WT and KD cells, results indicated reduced vimentin phospho-S72 expression without presence of FLNA **(Fig. 7F)**

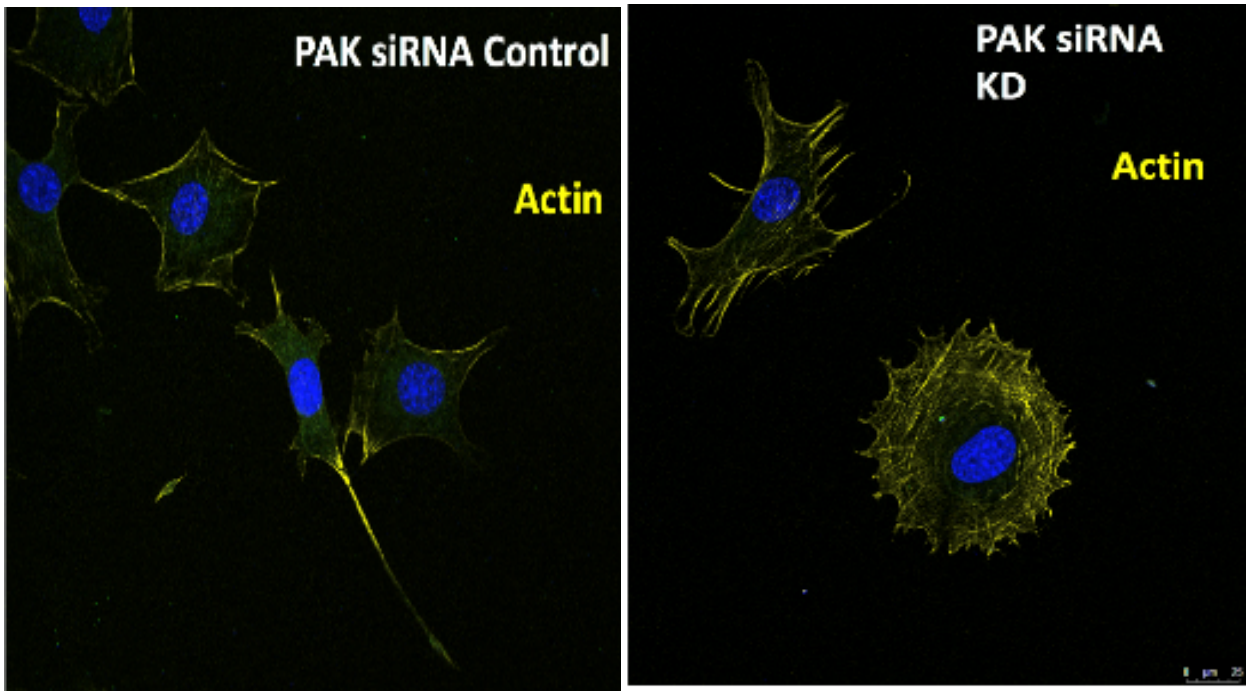
Figure 7

A

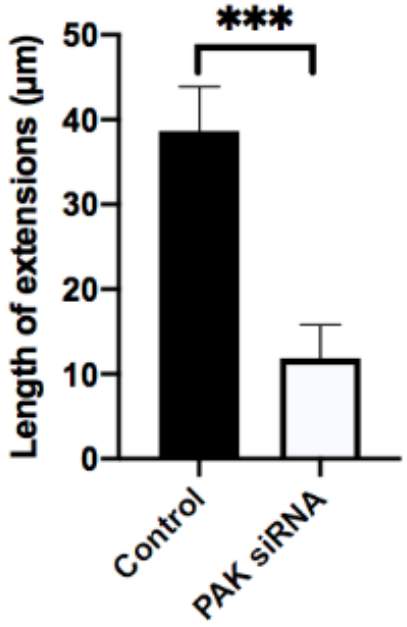

PAK siRNA
Control
PAK1
Control
PAK siRNA KD
PAK1
PAK SiRNA


PAK
SiRNA
+
PAK
SiRNA
-
PAK
61kDa

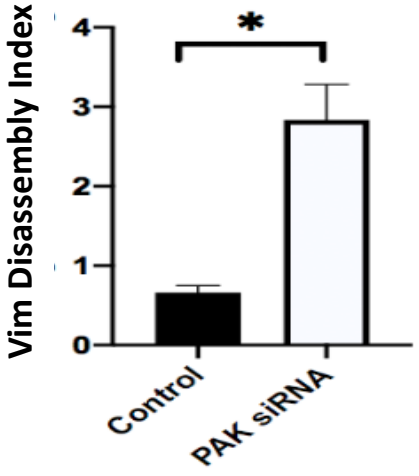
B


PAK siRNA Control
Actin
PAK siRNA KD
Actin

C


***
Length of extensions (µm)
50
40
30
20
10
0
Control
PAK siRNA

D


*
Vim Disassembly Index
4
3
2
1
0
Control
PAK siRNA

E

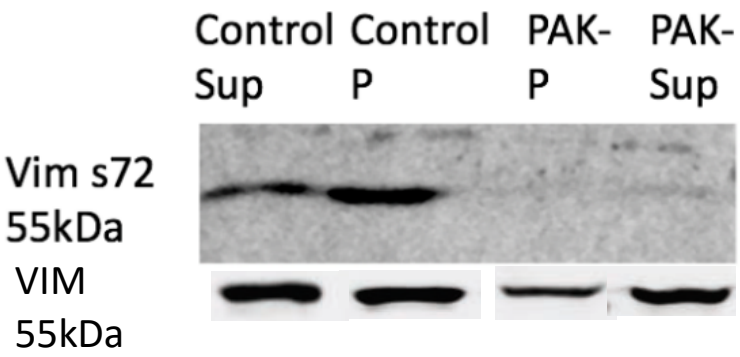


Fig. 7. PAK1 was transiently knocked down in FLNA WT cells with PAK1 siRNA. The effectiveness of knockdown was assessed by immunostaining and immunoblotting for PAK1 (**A**). The control group (left) was compared with the treatment group (right) for immunofluorescence intensity of PAK, PAK1 expression was also assessed and compared in PAK1 SiRNA untreated and treated cells(**A bottom figure**). Cells were also stained with phalloidin (for actin filaments) to assess cell extension formation after PAK1 knockdown. As shown in **B,C**, the control group (left) exhibited 4-fold longer cell extensions than the PAK1 siRNA-treated group (***$p<0.001$). (**D**) The vimentin disassembly index was computed, which was increased >5-fold by PAK1 knockdown compared with controls (*$p<0.05$). Western blot analysis was conducted for assessment of vimentin phosphorylation. Phosphorylated vimentin serine 72 was abundant in controls but was not detectable in the PAK1 knockdown group (**E**).

Actin-binding protein FLNA associates with PAK1

Co-immunoprecipitations of FLNA and PAK1 were conducted for assessing the potential association of PAK1 with FLNA in the context of PAK1 phosphorylation of vimentin. Immunoprecipitation of PAK1 followed by immunoblotting of the immunoprecipitates for FLNA, and immunoprecipitation of FLNA followed by immunoblotting of the immunoprecipitates for PAK1, showed association between the two proteins.

Figure 8

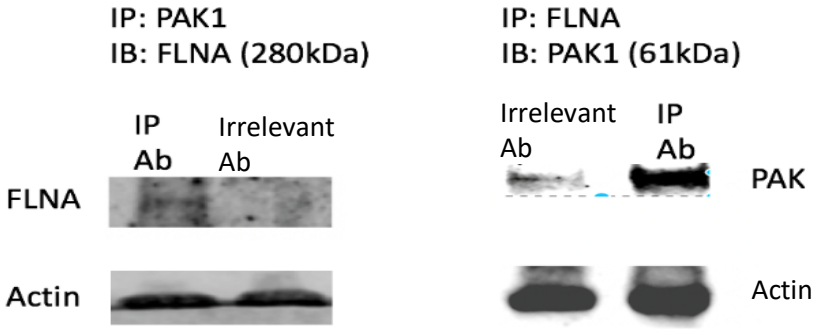


Fig 8. Left panel: Immunoprecipitates of PAK1 were immunoblotted for FLNA or with to an irrelevant antibody as control. **Right panel:** Immunoprecipitates of FLNA were immunoblotted for PAK1 or with an irrelevant antibody as control.

PAK1 directly phosphorylates vimentin at serine 72

I assessed the ability of PAK1 to phosphorylate vimentin using an in vitro kinase assay, which was performed with recombinant vimentin in the presence of PAK or PAK plus the PAK1 inhibitor IPA3. All vimentin samples were pre-treated with alkaline phosphatase to remove any background phosphorylation of vimentin prior to conduct of the assays. Addition of PAK1 to vimentin in the kinase buffer demonstrate increased phosphorylation of S72 compared to with controls in which PAK1 was not included. There was a 50% reduction of vimentin S72 phosphorylation when PAK1 was inhibited by IPA-3 (Fig. 9). These results indicate that PAK1 can directly phosphorylate vimentin S72 and that inactivation of PAK1 impacts phosphorylation of S72..

Figure 9

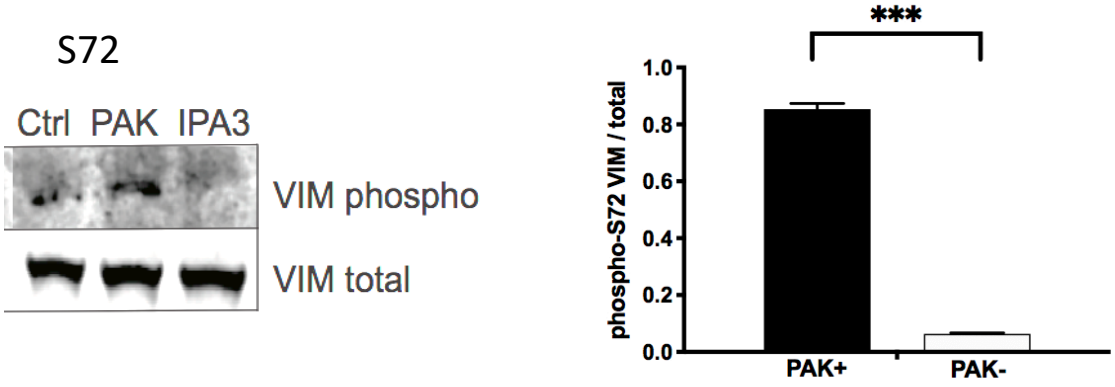


Figure 9
In vitro kinase assays were performed to examine PAK1-induced phosphorylation of vimentin. Recombinant VIM was treated with phosphatase buffer (to remove background phosphorylation of the substrate) followed by incubation in kinase buffer in the presence or absence of PAK1 and IPA3. The analyses showed that phosphorylation of vimentin serine 72 was strongly increased by PAK1 and that phosphorylation was reduced by 50% with the PAK1 inhibitor, IPA3.

VIII. Discussion

Previous data show that vimentin-dependent cell adhesion is enhanced by the expression of the actin binding protein FLNA (Kim et al., 2010a), but the possibility that vimentin and FLNA may interact to regulate the formation of cell extensions has not been considered. Here I established that FLNA plays a key role in controlling vimentin assembly from precursors (soluble protofilaments) and their conversion to insoluble, mature filaments. These data indicate that FLNA binds PAK1, which in turn phosphorylates vimentin, thereby promoting vimentin assembly. Taken together, these processes contribute to increased cell extension formation, which is critical for cell migration and matrix invasion by cancer cells.

FLNA has well-established, prominent roles in regulating the assembly of sub-cortical actin arrays and in controlling β1 and β7 integrin activation, processes that are important in cell migration (Li et al., 2010). In fibroblasts expressing FLNA, there were prominent and surprisingly elongated cell extensions, which were observed in fibroblasts with wild type levels of FLNA expression whereas in cells depleted of FLNA, the lengths and numbers of cell extensions were markedly reduced. Accordingly, FLNA expression strongly impacts the formation and elongation of cell extensions. FLNA contributes to the determination of cell morphology and accordingly is thought to play a role in invasive processes (Ji et al., 2018) (Jiang et al., 2013). An unresolved question with respect to FLNA is whether there is a functional interaction between FLNA and intermediate filament proteins, both of which play crucial roles in the mechanical stability of cells (Qin et al., 2009) and likely the stabilization of nascent cell extensions (Bertaud et al., 2010).

The type III intermediate filament protein, vimentin, is one of the classical markers of epithelial mesenchymal transition (Liu et al., 2015). My findings show that depletion of vimentin expression also impacts the formation of cell extensions in a manner that is phenotypically similar to depletion of FLNA. Notably, mesenchymal cell migration is highly dependent on the formation of cell extensions (Wang et al., 2019), which contrasts with the migratory morphology exhibited by epithelial cells (Campbell and Casanova, 2016). I found that transient knockdown of vimentin (but without altering FLNA) induces cell rounding, which is similar to that observed in FLNA KD cells. While the length of cell extensions was not influenced by vimentin knockdown, there was a large and significant reduction in the number of cell extensions per cell. These findings indicate that FLNA and vimentin may influence a common pathway that mediates the initiation of cell extension formation. While the expression of vimentin is positively associated with EMT-dependent behaviors such as the promotion of mesenchymal-type of migration by epithelial cells (Dongre and Weinberg, 2019), very recent data show that vimentin filaments can also reduce the motility of fibroblasts in three-dimensional collagen matrices (Patteson et al., 2019). While there are evident differences in the migratory behaviors and mechanics of vimentin-expressing cells in two-dimensions compared with three-dimensional matrices, it is likely that the formation of cell extensions depends strongly on the assembly of vimentin filaments in both migratory situations.

I found that vimentin filament assembly is markedly affected by the expression of FLNA. Based on western blot analysis, depletion of FLNA induces the collapse of vimentin filament structures and profoundly reduces the assembly of mature vimentin filaments. This blockade of maturation likely involves the retention of protofilaments as soluble units, which are then unable to assemble into insoluble, mature filaments. Notably, when vimentin filament assembly was inhibited by the selective inhibitors WFA and IDPN, similar alterations in the

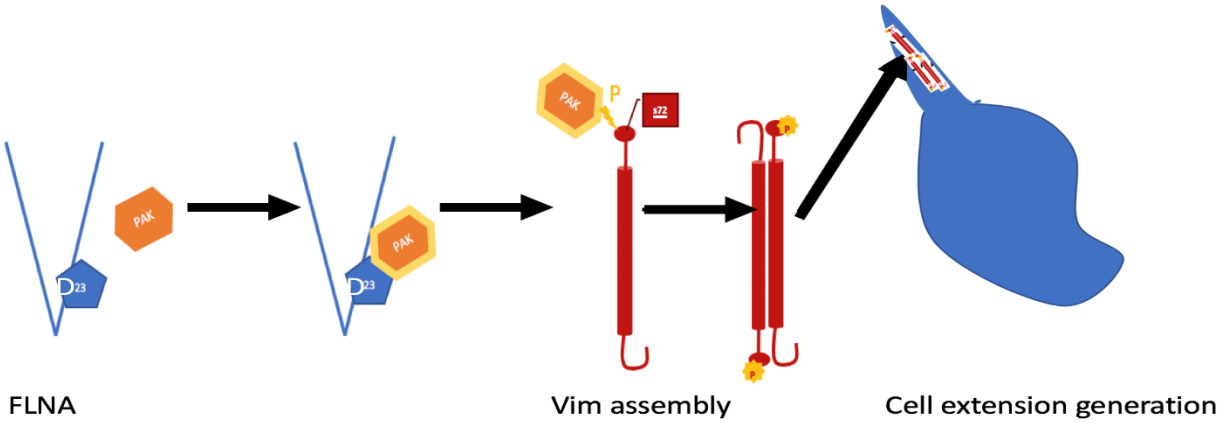
ratios of soluble and insoluble vimentin filaments were observed. Further, these alterations in vimentin filament assembly strongly affected the formation of cell extensions. Therefore, FLNA plays an indispensable role in facilitating vimentin assembly by promoting conversion of soluble protofilaments to mature insoluble bundles. These results are supported by and are consistent with earlier data demonstrating co-localization of FLNA and vimentin in mesenchymal cells and tumor cells (Brown and Binder, 1992) and evidence of reduced vimentin filament abundance in Src-transformed cells that interact with cells depleted of FLNA (Kajita et al., 2014). While these data advance the field it was important to determine the mechanism that links the functional activities of FLNA and vimentin.

With the use of *in silico* searches and inhibitor assays, I eliminated several possible candidates that have been shown earlier to impact the structure and functional activities of FLNA and vimentin ((Li et al., 2015);(Vadlamudi et al., 2002);(Ivaska et al., 2005);(Peverelli et al., 2018);(Sun et al., 2013)). I found that inhibition of the enzyme activity of PKC and PI3K minimally impacted cell extension formation while inhibition of ROCK activity strongly enhanced cell extension growth. Among those kinases that bound FLNA, only inhibition of PAK1 lead to blockade of cell extension formation. Further, immunoblot analysis showed that inhibition of PAK1 activity also caused increased the abundance of soluble vimentin precursors, suggesting that PAK1 is crucial for vimentin assembly. Consistently, cells exhibited large-scale retraction of cell extensions after transient knockdown of PAK1. Knockdown of PAK1 also reduced phosphorylation of vimentin serine 72, which is one of the key phosphorylation sites that regulate vimentin assembly (Eriksson et al., 2004). When vimentin serine 72 phosphorylation was assessed in FLNA WT and KD cells, there was increased abundance of phorspho-S72 in WT cells compared with KD cells. Taken together these data indicate that the FLNA-binding kinase PAK1, phosphorylates vimentin on serine 72, which promotes the

conversion of vimentin protofilaments into insoluble mature filament bundles, thereby contributing to the formation and stabilization of cell extensions.

IX. Conclusions

In fibroblasts, FLNA and vimentin cooperatively interact to promote the formation and elongation of cell extensions in a manner that is reliant on PAK1. FLNA binds PAK1, which then phosphorylates vimentin serine 72 and promotes the assembly of vimentin filaments. This process enhances cell extension growth, which is crucial in cell migration and the ability of mesenchymal tumour cells to invade the surrounding extracellular matrix.



X. Future Work

For defining in more detail how the proposed mechanism may influence tumour cell migration, additional experiments could be conducted in intact animal models of matrix invasion by cells. Complete knockout of vimentin in animals could trigger compensatory mechanisms by which cells increase the expression of other type III intermediate filaments, thereby complicating the interpretation of the importance of these filaments in cell extension formation and matrix invasion. Alternatively, tamoxifen-inducible, transient knockdown of vimentin could be performed in mouse models to obtain a better understanding of the role of vimentin in tumour progression and advancement.

Vimentin phosphorylation plays an important role in the assembly of vimentin filaments (Eriksson et al., 2004)but there are multiple phosphorylation sites in vimentin with poorly understood effects on vimentin filament assembly and the resultant effects on cell migration (Goldman et al., 1981). In addition to vimentin serine 72, phosphorylation of serine 39 and serine 56 could also be examined as these other sites may play critical roles in cell extension mechanism that is independent of PAK1 signaling.

Here I found that inhibition of ROCK activity induced the opposite effect on cell extension formation as did inhibition of PAK1 activity or PAK1 expression. Since inhibition of ROCK enhanced cell extension formation, ROCK may be associated with other important yet distinct mechanisms for controlling cell extension formation than does PAK1. Further, as ROCK and PAK1 are known to strongly affect the assembly of actin filaments in cell adhesion and migration ((Vadlamudi et al., 2002);(Yuda et al., 2018)), it will be important to define the impact of vimentin filaments independent from actin filaments in the process of cell extension formation.

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