The Role of β1-integrins In Centrosomal Stability

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Abstract/Resume

Centrosomes are major microtubule organizing centres that set up an internal microtubule (MT) network contributing to cell shape and to the formation of the mitotic spindle during cell division. Rearrangement of this MT array can be dictated by the centrosome and occurs during cell adhesion, polarization and migration. However, little is known about what regulates centrosome assembly and maintenance. β1-integrins are common cell surface receptors and we show that β1-integrin signalling is necessary for modulation of centrosome dynamics. In an attempt to identify the downstream components of β1-integrin signalling involved, we also discovered that the activation of focal adhesion kinase or integrin linked kinase are not required in maintaining centrosome integrity. This would indicate that a non-canonical signalling β1-integrin pathway might be involved in controlling centrosomal dynamics. This gives us greater insight into the mechanisms that control centrosomal stability and may lead to the better understanding of diseases like cancer and diseases, i.e. lissencephaly, which involve defects in cell polarization and asymmetric cell division, where the centrosome seems to have an important role.

Les centrosomes sont les principaux centres d'organisation des microtubules (MT) contribuant à la structure de la cellule et à la formation du fuseau mitotique au cours de la division cellulaire. Les centrosomes jouent également des rôles essentiels dans la réorganisation du réseau de microtubules nécessaire à l'adhérence cellulaire, l'établissement de la polarité et à la migration. Cependant

les mécanismes permettant l'assemblage et la maintenance des centrosomes sont peu connu. Nous démontrons ici que les récepteurs transmembranaires β1-intégrines sont nécessaires à la modulation de la dynamique des centrosomes. De plus, nos résultats visant à identifier les composants en aval de la signalisation β1-intégrine révèlent que l'activation des kinases d'adhérence focale ou kinases liées aux intégrines ne sont pas nécessaires dans le maintien de l'intégrité des centrosomes. Cela semble indiquer qu'une voie non-canonique de signalisation β1-intégrine pourrait être impliquée dans le contrôle dynamique des centrosomes. En conclusion les études présentées ici permettent une meilleure compréhension des mécanismes contrôlant la stabilité centrosomal. Ce qui en retour nous permet de mieux comprendre un grand nombre de pathologies humaines, telles que le cancer qui peuvent être notamment induites pas des anomalies de polarisation ou de division cellulaire asymétrique qui sont dépendants du bon fonctionnement des centrosomes.

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1 Literature Review

1.1 Centrosomes

Centrosomes are cellular organelles that have microtubule organizing capabilities[1, 2]. Each centrosome consists of a centriolar core which is surrounded by the pericentriolar matrix (PCM) (Figure 1A) and at interphase is often found adjacent to the nucleus (Figure 1B). This centriolar core comprises of a pair of centrioles which lie perpendicular to each other (Figure 1A). In vertebrates, centrioles are made up of 9 microtubule (MT) triplets that line up radially to form a barrel[3] (Figure 1A). The proximal ends of both centrioles in a centrosome are attached to each other[4] via rootletin, a large coiled-coil protein which belongs to the pericentrin family[5, 6]. At each distal end, plus ends of MTs attach which allow the centrosome to act as a basal body[7], similar to basal bodies seen in the formation of cilia and flagella which are projections at the cell surface membrane responsible for moving liquid past the cell surface and for cell motility. Centrioles duplicate during mitosis and are localized to spindle poles during cell division[8]. Hence, centrioles are thought to be important for cell division. However, it has been shown repeatedly that cells lacking centrioles can divide normally generating identical daughter cells[9-11]. Most plant cells and some animal germ-line cells lack centrioles and divide naturally by an alternative pole-based mechanism that does not require centrioles[12].

Spindle poles are found along the axis of cell division and extend MTs[13] which attach to each replicated sister chromatid at the kinetochore. These MTs

A

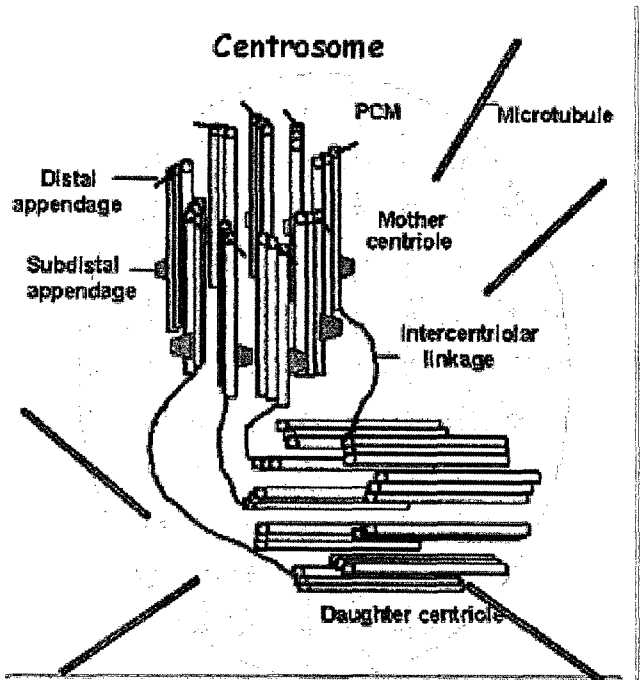

Image from: Crasta and Surana. Disjunction of conjoined twins: Cdk1, Cdeh1 and the separation of centrsomes. 2006. Cell Division.

B

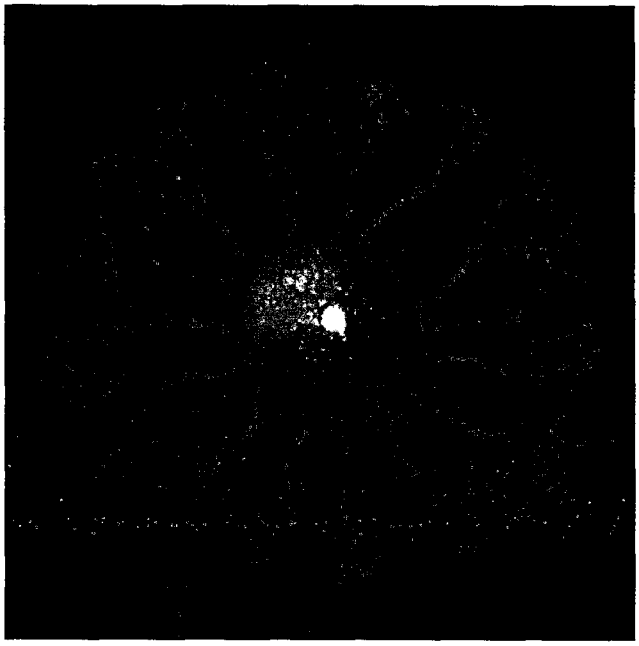

Figure 1: Structure of the centrosome.

Figure 1: Structure of the centrosome.

The centrosome comprises of a pair of centrioles, orthogonal to each other, which is surrounded by the PCM (A, centrioles in green and PCM in grey). Each centriole is made up of 9 MT triplets that lie in a barrel shaped configuration (A). The PCM is a matrix of proteins containing pericentrin and other centrosomal proteins (A). It is to the PCM that MTs attach via gamma-tubulin ring complexes and cofers the centrosome's microtubule organizing centre ability (A). The centrosome (B, in green) when visualized *in vitro* is observed adjacent to the nucleus (B, in blue) in an astrocyte attached to laminin (B, tubulin in red to show cell adherence).

emanating from the spindle poles may pull each chromatid to the opposite sides of a dividing cell[14]. This process ensures that all the genetic material in the cell is divided equally to form two identical daughter cells. The spindle poles also ensure genomic stability with the equal segregation of chromosomes during cell division, failing to do so leads to apoptosis and cell death.

In *Drosophila melanogaster*, flies lacking centrioles were able to develop normally to adulthood but died shortly after eclosion[10]. It appeared that centrioles were not essential for normal development of the fly to adulthood. However, after eclosion adult flies lacking centrioles did not survive as they lacked sensory cilia which require centrioles to form a basal body[10]. This discrepant requirement of centrioles in adult flies compared to embryos might stem from maternal contribution[15]. Maternal contribution of various proteins or transcripts might ensure the proper development of the embryo and the appearance of mitotic phenotypes might be due to the depletion of this maternal contribution[15]. With the lack of centrioles, it was interesting to observe the normal formation of mitotic spindles which would indicate that the centrioles are not essential in cell division in the fly. However, orientation of spindle poles for asymmetric division and completion of cytokinesis were disrupted when centrioles were absent[10]. Cytokinesis is the process where a furrow is formed to cleave the cell into two during cell division. While centrioles are dispensable for the formation of spindle poles and proper cell division, they have a role in asymmetric division, which is important in cell differentiation[10]. Asymmetric cell division generates two cells containing identical genetic material, with different physical characteristics, for

example, protein and RNA composition and localization. This process is responsible for cell differentiation in stem cells and gives rise to the different cell types seen within a multicellular organism.

The spindle poles that develop during mitosis form an axis through the cell which designates the orientation of cell division[16]. This axis is important for asymmetric cell division. In symmetric cell division, the cell membrane and cytosol are divided equally with no bias and hence daughter cells that arise from this type of cell division are identical in nature and behaviour. In contrast, asymmetric cell division occurs when the daughter cells that arise from cell division are not identical. This difference in daughter cells is usually due to each cell having different protein[17, 18] or RNA[19, 20] compositions and localization within the cytosol and at the cell membrane. The unequal inheritance of molecules from mother to daughter is a result of the orientation of the axis of the spindle poles[21, 22], amidst the non-homogenous distribution of proteins and RNA within the cell[17, 19]. Due to the accumulation of molecules, like Numb and Prospero in *Drosophila* neuroblasts[23], at specific locations in the mother cell during division, one daughter cell, but not the other, will inherit the accumulated molecules.

Asymmetric cell division is extremely important in cell differentiation. Stem cells often undergo asymmetric cell division so that their daughter cells inherit specific cues to differentiate and follow a particular cell fate[24]. This allows for cellular diversity such that cells may proliferate and differentiate to form the many different cell types populating an organism. In *Drosophila melanogaster* neuroblasts, asymmetric cell division generates a ganglion mother cell, which

proceeds to further divide to form neurons or glial cells, and a daughter cell that retains its neuroblast identity[25]. This asymmetry in cell types comes from distinct membrane domains in specific parts of the cell as well as the orientation of the spindle poles for cell division[26]. Such asymmetry ensures that only specific groups of proteins are inherited in the ganglion mother cell or in the daughter cell.

Marshall suggests that one purpose of the centrioles might be to recruit the proteins necessary for the PCM and to maintain its formation[27]. Centrosomal proteins might use the centrioles as a site of attachment and allow for the accumulation of centrosome-specific proteins at a specific locale in order to carry out its function[27]. The PCM is a fibrous lattice mesh structure which has pericentrin[28] as its primary component (Figure 1A). Other proteins such as γ-tubulin, in the form of γ-tubulin ring complexes (γ-TuRCs), interact directly with pericentrin. γ-TuRCs associate with and allow the negative ends of microtubules (MTs) to attach to the PCM from which MTs extend into the cytosol[13] (Figure 1B). This interaction between γ-TuRCs and the negative ends of MTs anchors MTs to the centrosome and allows its arrangement in the cytosol to form the stable scaffold required to give a cell its shape and to direct movement via rearrangement of this cytoskeleton. Some other proteins like Aurora A[29, 30], integrin-linked kinase[31] (ILK) and Polo-like kinase 1[32] (Plk1) are also known to associate with the PCM and are involved in a variety of signalling pathways that will be discussed later.

The MT network that is set up by the centrosome also creates tracks by which proteins and other cargo often packaged within vesicles may be transported

from one part of the cell to another[33]. In fact, transport proteins like dynein[34] and dynactin[35] have been found at the centrosome. These motor proteins are well known to load and transport cargo within the MT network by "walking" on the MTs[36-38]. This is expected since the centrosome is a well known MT organizing structure and may act as a hub within the cell to organize the logistics of cargo trafficking. It is also through this trafficking network that centrosomal proteins are delivered to their final destination where they can act and have a hand in the construction of the centrosome[39, 40].

1.2 Centrosomes in Mitosis

During mitosis, centrosomes replicate with the cell cycle in distinct stages. First, centrioles disengage and separate to form a mother and daughter centriole[3, 41]. Each mother and daughter centriole then undergoes duplication and elongation to each form a pair of centrioles[3, 41]. Each pair of centrioles then mature and separate, migrating to opposite ends of the spindle poles during mitosis from which MTs extend[13] to attach to the kinetochore of the chromosome and pull each sister chromatid into opposite ends of the cell[14]. This ensures proper chromosomal segregation and the integrity of cell division. These stages synchronize precisely with different stages of cell division[41]. Centriole disengagement coincides precisely with the G1 phase of mitosis, duplication and elongation of centrioles with the S-G2 phase of mitosis and finally maturation and separation of mother and daughter centrosomes with the M phase of mitosis[41].

This synchronicity of the centrosome and cell cycle seems to indicate a possible role of the centrosome in cell cycle regulation. It has been shown that many proteins that localize to the centrosome are important for cell cycle progression and the activation of checkpoint responses[32, 42, 43]. Cell cycle progression is a highly regulated process. To transit from one stage to the next requires the cell to achieve specific characteristics before advancing[44]. For example, prior to mitosis, the cell has to "check" that the spindle is properly assembled before proceeding. This requires the activation of specific signals and the inhibition of others, which include the activation of checkpoint kinase 1 and the inactivation of Plk1 at the spindle assembly checkpoint[42, 43]. If a cell is not ready to progress to the next stage, these checkpoint signals are not activated which results in cells arresting mitosis and waiting for conditions to be favourable before moving on[44]. With each stage of the centrosome cycle coinciding with different stages of mitosis, the different characteristics of centrosomes at each stage of the cycle might possibly act as a checkpoint for mitosis as well as localize the factors that govern the admittance of the cell into the next stage of mitosis. This would strongly implicate the centrosome in cell cycle progression.

Neurogenesis in vertebrates also relies on asymmetric cell division to populate the neocortex with neurons and glia during development[45]. The orientation of the spindle poles during neurogenesis is important[46]. A horizontal axis with respect to the basement membrane of the neuroepithelium for division in neural progenitor stem cells allows for proliferation and self renewal by generating two identical neural stem cells, while a vertical spindle pole axis,

parallel to the basement membrane, would give rise to a neural progenitor stem cell and a neuron or glial cell and this allows for differentiation[46, 47]. Perturbations in the orientation of this axis have shown to lead to overproliferation of neural progenitor stem cells and even tumour formation in *Drosophila*[48, 49]. Hence, the positioning of the spindle poles during mitosis is vital for proper cell division.

1.3 Centrosomes at Interphase

At interphase, centrosomes are usually located adjacent to the nucleus and are known as major microtubule organizing centres[1, 2] (MTOCs). The MTs anchored to the centrosomes extend into the cytosol to form the cell's cytoskeleton. The dynamic meshwork of MTs and actin filaments organized by the centrosome provides a support upon which the cell may maintain or change its shape. Maintaining cell shape is important for its function. For example, neurons need to be able to form axons and dendrites, specialized processes from the cell body, so that they may establish connections with other neurons to receive and transmit signals to and from the brain.

Changes in cell shape are also crucial for cell motility and polarization. These changes are often dictated by a family of small Rho-GTPases, like Rac, Rho and Cdc42. These small molecules control the formation and dynamics of the cytoskeletal network[50-52]. The activation of this family of Rho-GTPases involves the addition of GTP to the protein by guanine exchange factors that replace GDP with GTP within the protein[53]. Guanine activating proteins proceed to dephosphorylate GTP to GDP within the Rho-GTPase thereby deactivating it[54].

Rho-GTPases have been implicated in both actin and microtubule dynamics, with a significant body of knowledge devoted to their role in organization and formation of actin filaments into lamellipodia, actin projections that form a mesh located at the mobile edge of a cell, and filopodia, slender finger-like projections similarly found at the cell membrane[55, 56]. Both lamellipodia and filopodia are commonly thought to provide the necessary traction during cell migration, spreading and adhesion[57]. These changes at the cell periphery are also coupled with changes to the MT network within the cytosol. In combination, these changes to the actin and MT framework in the cell can result in changes in cell shape that not only contribute to motility, but to cellular function as well. The MT network is reorganized through depolymerisation and repolymerization of MTs in the cytosol and is integral in the formation of processes and extensions of the cell during adhesion and migration[58]. With the high involvement of MTs in these processes and the MTOC abilities of the centrosome, changes to the MT and actin cytoskeleton organization are often thought to be orchestrated by the centrosome.

1.3.1 Cell Adhesion

When cells come into contact with the extracellular matrix (ECM), cell surface receptors like integrins and dystroglycan bind to their ligands within the ECM, which activate downstream targets like Rac, Rho and Cdc42, among other signalling pathways[59, 60]. Activation of these proteins lead to the restructuring of the MT and actin cytoskeleton and the formation of lamellipodia at the edges of

the cell periphery in contact with the ECM[55]. This allows the cell to spread and form strong contacts with its substrate which is apparent when astrocytes adhere to ECM proteins *in vitro*. *In vivo*, cell adhesion is important for astrocytes to be able to send out processes and contact neurons to serve in their supportive roles. Also, cell adhesion is required for the migration of cells throughout an organism. Providing cells a substrate to adhere to and move on would allow them to reach their final destination and carry out their specified functions. For example, neuroblasts have to migrate through the cortex to reach their final cortical layer where they make the necessary connections for proper brain function[61]. The neural network that is set up during nervous system development involves each axon connecting with their specific targets[62]. Axonal guidance is a highly specialized process that requires the adhesion and extension of a cellular process in the direction of its target[63]. Directionality of the axon is determined by the secretion of a variety of different factors, including semaphorins[64] and netrins[65]. These factors have been shown to change the shape of the growth cone of an axon[64, 65] which is established by the centrosome-controlled MT network within the axon[66].

The physical modifications that take place in an adherent cell involve extensive changes in cell shape particularly during cell spreading after initial contact as reported in endothelial cells by Nobes *et al*[55]. In some respects, astrocytes behave similar to endothelial cells in suspension. Cells are round in suspension but upon contact with a rigid substrate, they reorganize their MT network to spread the cell on the substrate[67]. Astrocytes are also observed to

extend processes upon spreading and this requires the generation of MTs and rearrangement of the actin filaments to create the force necessary for the protrusion to form[58, 68]. With the changes in the MT array observed during cell adhesion and spreading, it would be expected for the centrosome to be involved.

1.3.2 Cell Polarization

Cell polarity is important to create distinct specializations within different membrane and cytosolic domains for proper cellular function[69]. For example, cells polarize during division so that they may divide asymmetrically[70]. Epithelial cells are polarized to ensure that the different membrane domains of the cell possess specific characteristics, i.e. the apical versus the basolateral membrane, like the ability for unidirectional transport of substances through the cell[71]. During cell migration, the cell moves in a specific direction which would create a leading and trailing edge of the cell[72-75]. This difference in characteristics between the leading and trailing edge of the cell gives it polarity[74, 75]. During cellular migration, these polarization events have been well characterized in astrocytes and fibroblasts[76-78].

Polarization events during cell migration are commonly assayed in the scratch wound assay[79]. This assay involves making a scratch through a confluent monolayer of astrocytes or fibroblasts[78]. The scratch initiates cell polarization events in cells adjacent to the wound site and these cells would eventually migrate directionally into the wound site[78]. These events include the reorientation of the centrosome and Golgi apparatus "woundside" in cells directly adjacent to the

scratch[78]. A MT-rich process is then extended into the wound site which initiates the migration of astrocytes or fibroblasts into the wound[78].

The family of Par proteins have been heavily implicated in establishing cell polarity by segregating and localizing to specific areas within the cell[80]. In particular, Par6 has been shown to be recruited to the apical surface of the cell membrane and tight junctions upon activation of Cdc42[81]. In a monolayer, the apical surface is considered the side of the cell that is not in contact with the substrate, while tight junctions are located at cell contacts within a monolayer. The accumulation of Par6 to these domains in the cell further recruits atypical protein kinase C (aPKC) and Par3 proteins[81]. Other Par proteins like Par1 not only localize to the lateral membrane of epithelial cells[82], but also remove Par3 proteins from areas of the cell lacking Par6[83]. This segregates the cell membrane into different compartments containing specific proteins. It is believed that this compartmentalization of proteins gives the cell its polarity by conferring distinct characteristics at the apical, lateral and basal domain of the cell surface.

Cell polarization gives directionality to a cell for movement. The spatial differences within a cell in terms of protein localization at the cell membrane create micro-domains such that the proteins localized within these domains exert only isolated effects at their targeted areas. For example, proteins located at the trailing edge of a moving cell would be enriched in proteins involved in the breakdown of adhesive structures, while the formation of adhesive structures would be required at the leading edge, thereby pulling a cell forward[72, 75]. Also, opposing MT dynamics are observed in different parts of a moving cell. The

leading edge requires the distribution of specific proteins like integrin-mediated protein kinase A and profilin for MT polymerization[84, 85]. Proteins for MT depolymerisation are required at the trailing edge; for example focal adhesion kinase (FAK), PDZ-Rho GEF and Rho effector Rho kinase II (ROCKII)[84-88].

Characteristic events that occur in polarized astrocytes during the scratch wound assay are the reorientation of the Golgi apparatus and the centrosome "woundside" which has been shown to be regulated by extracellular signal-regulated kinase (ERK)[89], a downstream target in integrin signalling[90]. A protrusion is then extended into the wound with the centrosome and Golgi apparatus at its base[78]. This process extension requires the rearrangement of MTs for its formation which is driven by the activation of Rho-GTPases[87, 91]. Drugs like nocodozole and colchicine which disrupt MTs and MT dynamics have been shown to also disrupt process extension[66, 92]. Also, perturbation of Cdc42 which controls actin dynamics disrupts both process formation and centrosome reorientation during cell polarization[78, 93, 94]. However, an interesting finding was that disruption of Rac activity, another Rho GTPase similar to Cdc42, only inhibited process extension, leaving centrosomes to reorient normally woundside[95]. Hence, it should be noted that a cell is said to be polarized depending on the position of its centrosome and Golgi apparatus and that cell polarization can occur in the scratch wound assay without the extension of a process[91]. It is likely however that the centrosome normally also plays a major role in this process extension since one of its main functions is to organize the MTs within a cell. The nucleus may then be pulled into this MT-rich process

which is necessary for the migration of the cell into the wound site or during neurogensis[93, 96].

Cdc42 and Rac have been implicated in the processes of cell adhesion and polarization. Activation of these small Rho-GTPases is triggered upon the engagement of cell surface receptors, like integrins, by their ligands on either the ECM or an adjacent cell's surface[59]. This shows the importance of cell-matrix and cell-cell interactions to modulate the plethora of signalling cascades that control the physical characteristics and behaviour of a cell. Since the behaviour of the centrosome is also precisely coordinated during these processes, the activation of these signalling processes during initial cell receptor engagement might be responsible for the observed changes in centrosomal dynamics.

1.3.3 Cell Migration and Motility

During cellular migration, the centrosome has been observed to localize toward the leading edge of the moving cell[97]. In migrating neuroblasts, a process is extended in the direction of the movement and the centrosome has been often observed to localize to the base of that process[98]. This is followed by the movement of the nucleus which seems to follow the centrosome into the extended protrusion[99, 100]. It was thought that the centrosome acted as a steering mechanism and chose the direction in which a cell moved[101, 102]. However, it has been shown that nuclear movement is independent of the centrosome[103] and that the centrosome does not dictate direction in which a cell moves, but rather stabilizes the chosen direction[104]. It is hypothesized that this stabilization is achieved by the

centrosome's ability to control MT dynamics and anchor the microtubular network to stabilize movement in a given direction[104, 105]. It is these MT dynamics that give the cell traction on its substrate and generates enough force for it to move.

Another interesting hypothesis is that the MTs might have a role in pulling the nucleus through the cell during migration[106, 107]. As mentioned previously, the MT network is used in transport of proteins and organelles within the cell. Similarly, the nucleus might be transported this way during nuclear migration. Nuclear migration is not only important during migration of the cell, but also to position the nucleus specifically within the cytosol for proper functioning[96]. In neurons, the position of the nucleus contributes to specific neuron morphologies which are important for setting up proper brain architecture and allows neurons to tile and make proper connections[108]. Nuclear migration defects have been observed in lissencephalic (smooth brain) patients suggests that nuclear position is vital for proper migration of neurons in the developing brain[107]. Recent work has shown that the centrosome is linked to the nucleus via interactions of nuclear lamins between the PCM and the nuclear envelope[109]. This directly tethers the nucleus to the centrosome and in turn[110] MTs, which might allow for nuclear transport.

Changes in the MT and actin network also contribute to the movement of growth cones during axonal guidance[111]. It has been reported that the centrosome is seen at the base of nascent axons which would indicate a role for the centrosome in coordinating the MT network required for the axonal projection[98].

This is important for neurons to guide their axons so that they may find their specific target and form the connections required for neural networks to carry out their functions.

1.4 Centrosomal Signalling

The centrosome has also been heavily studied in multiple signalling pathways. Aurora A, a serine/threonine kinase, is localized to the centrosome and is implicated in spindle body formation during mitosis[30, 112]. Cells overexpressing Aurora A show centrosomal amplification and multi-polar spindle formation which indicates its function in centrosomal maintenance and segregation[113-115]. In fact, activation of Aurora A has been implicated in spindle assembly as it was observed to regulate MT nucleation by recruiting the factors necessary for the formation of asters, the MT extensions projecting from the spindle poles responsible for separating chromosomes during mitosis[116].

Another protein that is also localized to the centrosome is ILK[31]. ILK or integrin linked kinase is commonly found in focal adhesions and it physically interacts with the cytoplasmic domain of β-integrins[117, 118]. ILK is a protein that is well known in focal adhesion signalling and has been shown to be activated upon cell adhesion where it transduces signals for integrin signalling activation which will be discussed later[118, 119]. In the centrosome, ILK seems to have a novel role in the organization of spindles during mitosis and inhibiting ILK disrupts proper spindle formation[31]. The Dedhar lab showed that a consequence of ILK inhibition is Aurora A inhibition. Aurora A activity has been previously shown to be very

important for proper centrosomal function[31]. Plk-1 is another example of a centrosomal protein and has been implicated with β1-integrins in carcinoma invasion of tissue[120]. The function of Plk-1 in the centrosome seems to be to recruit γ-TuRCs so that MTs may anchor to the centrosome[121, 122]. It has also been shown to regulate localization of Aurora A to the centrosome[123].

1.5 Integrins

A protein family that has been implicated in cell adhesion, migration and polarity are the integrins. Integrins are αβ heterodimers that are known to have both structural and signalling roles[124]. There are 18 known varieties of α-subunits with 8 known β-subunits[125]. Different combinations of α- and β-subunits produce 24 known integrins, with each heterodimeric combination of integrin having specific ligands and functions[125]. As an example, α1β1 is known to engage laminin and collagen[110, 126] and has been found to be expressed in neurons and implicated in the formation of point contacts which are important in growth cone motility[127-129]. ECM proteins like laminin, fibronectin and vitronectin are common receptors for many integrins[130-132]. Upon binding their corresponding ECM ligands, integrins are known to cluster and form focal adhesions and point contacts[129, 133], with point contacts being approximately 10 times smaller than focal adhesions[134-136]. Both types of adhesive contacts are associated with a complex of proteins that links the ECM to the actin cytoskeleton and activates a plethora of signalling pathways which have many effects on protein expression and cell behaviour. In addition to ECM proteins, integrins have also been shown

to interact with cell adhesion molecules like N-cadherin where they mediate similar functions to that of ECM protein interactions[137].

1.5.1 Focal Adhesions

When focal adhesions are visualized by immunocytochemistry, they appear as dash-shaped protein aggregations at the cell surface in contact with a substrate (Figure 5A, top row left two panels). These protein complexes are usually seen throughout cells that have adhered and bound to substrates like laminin and fibronectin (Figure 5A, top row left two panels). Integral proteins found within some focal adhesions are the β1 integrins, α5β1, α6β1 and α7β1 to name a few [125, 136] and the β3 integrin, αVβ3[138]. These integrins are known to have both structural and signalling roles within focal adhesions. Integrins span the cell surface membrane to form a physical link between the actin cytoskeleton and ECM and are also detectors and transducers of environmental signals[125, 133, 139].

Extracellularly, integrins within focal adhesions engage ECM proteins, like fibronectin, laminin, etc., before interacting with talin[133], paxillin[140, 141] and vinculin[133] intracellularly. This bridge between the external and internal environment of the cell allows it to adhere, maintain a specific morphology and move on different substrates. The formation of these adhesive structures at the leading edge of a moving cell, coupled with the release of adhesions at the trailing edge, allows cells to move across substrates[142]. In addition, these protein complexes activate different signalling cascades, some of which will be discussed later[125]. These pathways are known to direct protein transcription[143], cytoskeletal

dynamics[59, 144] and even cell cycle regulation[90, 112, 117]. Some signalling molecules shown to localize to focal adhesions are FAK and ILK, as mentioned previously.

1.5.2 Point Contacts

Point contacts are smaller than focal adhesions and form a punctate staining pattern at the cell surface[136]. α1β1 integrin is the primary integrin heterodimer found in point contacts[135, 136]. Interestingly, it seems that focal adhesions are borne from the clustering of point contacts[136]. This phenomenon is observed in astrocytes where upon initial attachment, astrocytes rapidly form point contacts containing α5β1, α6β1 and α1β1 integrins[136]. However, those containing solely α5β1 and α6β1 integrins further aggregate to form focal adhesions[136]. The remaining point contacts do not undergo anymore clustering and remain as punctate structures at the cell membrane[136].

Likewise, proteins like paxillin and vinculin have been shown to localize heavily to point contacts in growth cones[133], in addition point contacts in astrocytes have been shown to contain clathrin[135]. Within point contacts, integrins may perform a similar function for structural integrity in creating a physical bridge that joins the external ECM to the internal actin cytoskeleton[135]. Since these point contacts form rapidly, it is thought that these adhesive structures form to first bind the cell to a substrate and start the adhesion and cell spreading process, while focal adhesions formation occurs subsequently to strengthen adhesion and initiate different signalling pathways[136]. In astrocytes, point contacts are observed to form at the cell periphery prior to the formation of focal

adhesions[136]. Point contacts also activate signalling pathways. It has been reported that RhoA and RhoB localize to point contacts which would imply the activation of Rho signalling from point contacts[145]. In addition, FAK was reported to localize to point contacts in growth cones and is essential for growth cone guidance[146]. It has been hypothesized that the activation of these signalling pathways play a role in axon pathfinding[145]. However, little is known about other signalling pathways that might be activated exclusively from point contacts.

1.6 Integrin Signalling

Along with their structural roles, integrins are well known to activate a variety of different signalling cascades from both focal adhesions and point contacts[59, 146]. These pathways regulate apoptosis[147], cell differentiation[148] and migration[149] which change the behaviour of a cell depending on its environment.

1.6.1 Focal Adhesion Kinase

FAK is a very well known signalling molecule that is found in abundance at both focal and point contacts[146, 150]. FAK has been shown to bind the cytoplasmic domain of β-integrins *in vitro*[140]. Upon focal adhesion formation and the engagement of β-integrins to their ligands, FAK is activated by the phosphorylation of tyrosine-397[151]. This phosphorylation event triggers the activation of signalling cascades that are downstream from FAK[151]. These include the Rho-GTPases, Cdc42 and Rac, and the mitogen-activated protein (MAP) kinase pathway[152]. The Rho GTPases control actin dynamics and the formation of

lamellipodia and filopodia at the leading edge of a moving cell[52, 91, 153]. Motile cells undergo changes in cell shape which are brought about by changes in the MT array within a cell to accommodate the movement along a substrate[86]. Hence, with the ability to organize the MT network within a cell, the centrosome might be implicated in coordinating these changes.

FAK seems to be important in cell adhesion and migration as cells lacking FAK are slow to spread upon attachment and also show decreased migration[154, 155]. The kinase activity of FAK is vital to these processes as rescuing FAK-deficient cells with a kinase dead FAK showed no improvement of the phenotype[154, 156, 157]. It is presumed that the observed impediment to spreading and reduced migration of cells with FAK inhibition is due to the disruption to signalling proteins downstream from FAK. During migration, focal adhesions are assembled at the leading edge and broken down at the trailing edge of the cell[88, 158, 159]. FAK has been implicated in this focal adhesion turnover[160], hence perturbing FAK function leads to disruption of cellular migration, with further evidence pointing in particular to the changes in Rho activity as the main cause of this effect[153].

However, an interesting result is that FAK-null flies are viable and develop without obvious anomolies[161]. They do not exhibit any defects in integrin signalling and cell adhesion[162]. FAK-null mice show embryonic lethality with severe adhesion defects[149, 163]. These opposing results might indicate a divergence of FAK requirement during development between mice and flies and may arise due to compensatory mechanisms that are present in flies compared to mice which would explain the absolute necessity of FAK in mice and not flies. In other

Drosophila results, it has been shown that FAK deficient flies exhibit abnormal optic stalk development and glial function[162, 164] as well as faulty synaptic transmission and axonal conduction[164]. Also, with FAK being an upstream activator for the MAP kinase pathway, the loss FAK function leading to MAP kinase suppression causes the overgrowth of neuromuscular junctions in flies[165]. While FAK seems to be non-essential to flies during its development, this protein is still important for the proper functioning of the organism. Interestingly, Pyk2, a FAK-related protein tyrosine kinase, has been reported to compensate for loss of FAK function. Activation of Pyk2 is often observed to be upregulated when FAK activation is disrupted and this might obscure the true function of FAK[166, 167]. Consequently, we should be cautious when we proceed with investigating the role of FAK in these model organisms and the conclusions that may be drawn from the results with relevance to humans.

1.6.2 Integrin Linked Kinase

ILK is another protein kinase that has been shown to interact with the cytoplasmic domain of β-integrins[168]. Similar to FAK, ILK is also activated upon clustering when focal adhesions are formed and their β-integrins engaged[118, 169]. Both ILK and FAK have been shown to be important intracellular integrin binding proteins that facilitate the activation of some common downstream pathways like the Rho-GTPases[170, 171] which regulate changes in cell morphology as dictated during cell adhesion[59]. Other downstream targets of ILK include the protein kinase B signalling pathway which appears to be exclusively activated

through ILK and not FAK[172, 173]. This pathway is involved in cell survival and proliferation[174].

Recent work in Dr Dedhar's lab has shown that ILK is required for the proper alignment of the spindle poles in cells undergoing asymmetric cell division[31, 117]. Disruption to ILK function has shown abnormal mitotic spindle assembly and subsequent failure of DNA segregation during cell division[31]; this shows β1-integrin signalling as possibly having an effect on centrosomal dynamics since integrins are the most well known activator of ILK. Interestingly, they show that ILK is localized to interphase centrosomes in human endothelial kidney 293 (HEK293) cells[117]. However, in our results, astrocytes showed no localization of ILK at the centrosome (Figure 8, bottom leftmost panel). This might be a cell specific phenomenon and hence, another objective would be to determine whether β1-integrin signalling is an astrocyte specific requirement for centrosomal stability.

1.6.3 Other Proteins and Pathways

In addition to FAK and ILK, Yang and colleagues showed that activated Cdc42-associated kinase-2 (ACK2) is rapidly activated upon engagement of integrins through fibronectin, as well as when cells are attached to polylysine via charge interactions[144]. This in turn highly activates Cdc42 which has been mentioned previously to be important in cytoskeletal dynamics and plays an important role during cell adhesion and migration. C-Jun kinase (JNK1) was also shown to be activated by ACK2[143]. JNK1 is implicated in a variety of cellular

functions like cell proliferation, apoptosis and differentiation[175]. Hence, its activation has far reaching consequences for cellular behaviour. ACK-2 is not the only regulatory protein upstream from JNK1. The MAP kinase pathway was also shown to activate JNK1 and is similarly activated by engagement of integrins[176]. Other effects of MAP kinase include increased cell proliferation and regulation of protein transcription and localization[177, 178].

1.7 Previous Work

As mentioned previously in Section 1.3.2, the scratch wound assay has been used successfully by Etienne-Manneville and Hall to investigate the role of Cdc42 in cell polarization events[78, 179], thereby implicating integrins in the process. Using this model, previous work in the Carbonetto lab investigated the role of dystroglycan (Dg) and β1-integrins in the scratch wound assay. Dg is a ubiquitous cell surface protein in the nervous system and muscle that is integral in dystrophin-associated glycoprotein complex[180, 181]. Similar to integrins, Dg participates in a complex which enables the adhesion of a cell to a substrate via binding to ECM proteins like laminin[180, 181]. This made both integrins and Dg likely candidates in the process of cell polarization. Results showed that astrocytes null for Dg or β1-integrin did not extend processes into the wound site when a scratch was made indicating a possible role for Dg and β1-integrin in cell polarization[182]. When stained for MTs, astrocytes lacking Dg or β1-integrin showed the misorientation of MTs[182]. In wildtype astrocytes, MTs were observed to be oriented along the length of the process as it extended into the wound,

however, this orientation was lost in Dg-null and β1-integrin null astrocytes[182]. Loss of specific MT orientation prevents the formation of the protrusion required for cell migration[182]. This led to the question of whether process extension was inhibited due to cells being unable to polarize or dysfunction in MT organization. Hence, using the centrosome as a landmark to show cell polarization through its reorientation, cells were stained with two major centrosomal proteins, pericentrin or γ-tubulin. Interestingly, staining for the centrosome in β1-integrin null astrocytes, but not in Dg-null astrocytes, showed a disruption of centrosomal integrity (unpublished; Peng). Astrocytes lacking Dg still had intact centrosomes with only cell polarization of the centrosome partially disrupted[182]. Localization of pericentrin and γ-tubulin into a distinct focus is indicative of an intact centrosome, however, both pericentrin and γ-tubulin appeared diffuse throughout cells lacking β1-integrin and no distinct foci of either protein was observed (unpublished; Peng). Furthermore, similar results were obtained when β1-integrin function was blocked using an antibody. This implicated β1-integrin in the maintenance of centrosomal stability and revealed a novel pathway that seemed to control centrosomal integrity. It also gives us a way to disrupt the centrosomes so as to investigate the role of the centrosome in different cellular functions.

1.8 Objectives and Rationale of Work

The literature on centrosomes and integrins reflect some common themes between them, with both playing important roles in cell polarization and migration. This investigation suggests that the relationship between the

centrosome and integrins might be even closer than previously thought. We have shown that integrin signalling in astrocytes is important for centrosome assembly and maintenance. Specifically, a candidate emerges in the form of β1-integrin signalling. We aim to further identify downstream signalling components involved in centrosomal stability. Better understanding of the centrosome will also reveal the role of this organelle in different cellular behaviours like polarization and migration.

In addition to studying cell polarization, the scratch wound assay with astrocytes has been used to model the formation of the astro-glial scar after central nervous system injury since the events that follow the scratch mimic events observed *in vivo* after nerve trauma[183, 184]. The astro-glial scar creates a non-permissive environment for axons to reinnervate the trauma site[185]. This involves both the polarization and migration of the adjacent astrocytes into the wound thereby preventing axons from remaking their previous connections to re-establish connectivity. Hence, understanding the role of the centrosome in this process would give insight into how therapeutics might be designed to allow for neuronal regeneration in the central nervous system after injury. Cell polarization and migration are not only important in astro-glial scar formation, but also during neuronal migration in the developing embryo. The brain architecture is important for neurons to set up the connections necessary to ensure proper brain function[186]. The patterning of the brain architecture requires the controlled migration and morphology of neurons to their final destination so that appropriate connections

may be established[186]. Without proper patterning, brain architecture is disrupted and its function lost.

The disruption of the integrity of the centrosome, resulting in the centrosome no longer retaining its single distinct structure adjacent to the nucleus in interphase cells, is observed in multiple cancer cell lines where these cells contain supernumerary centrosomes[187, 188]. In order for these cell lines to proliferate, the multiple centrosomes have to coalesce such that proper cell division can occur with a single pair of mitotic spindle poles[189, 190]. Studying the signalling pathways involved in centrosomal stability will give great insight into what controls aspects of the fundamental behaviour of this organelle. This research might also shed some light on how supernumerary centrosomes function in cancer cell lines during mitosis for the development of therapeutic strategies.

1.9 Summary of Results

The results from this study show that astrocytic centrosomes disassemble quickly upon detachment from their substrate, fibronectin, and assemble rapidly upon attachment to laminin, fibronectin, collagen and polylysine. β1-integrin signalling is necessary for maintaining centrosomal integrity, with cell adhesion alone insufficient, and centrosome disruption was observed upon disruption of integrin signalling. We were able to eliminate both FAK and ILK as downstream components of β1-integrin signalling involved in centrosomal maintenance. This was achieved by knocking down FAK activation via a FAK-related non-kinase (FRNK) dominant negative construct and a small molecule inhibitor, PF-228

(courtesy of Dr Tony Parsons, Pfizer) and staining for the centrosome to check centrosomal integrity. Similarly, total ILK expression has been knocked down using plasmid ILK siRNAs (ILK782 and ILK285) and Stealth oligomer ILK siRNAs (from Invitrogen). These knockdowns showed no disruption in centrosome structure, indicating that activation of FAK or ILK alone is not solely responsible for maintaining centrosomal integrity. Finally, using Secramine A, we were able to tentatively absolve Cdc42 from participation in centrosomal dynamics since no effect was observed upon application of the small molecule inhibitor. However, further confirmation of actual Cdc42 inhibition is pending.

Having a better understanding of the factors that control centrosomal stability will give insight into the importance of the centrosome in different cellular events, i.e. cell polarization and migration, as it allows us a way to disrupt the centrosome and observe the consequences of doing so. A disrupted centrosome is often seen in neuronal developmental defects and cancer. Knowing the signalling pathways involved with this phenotype will contribute to the better understanding of the disease mechanism and help in developing therapeutic strategies to combat these debilitating diseases.

2 Materials and Methods

2.1 Astrocytic Cell Culture

Primary rat astrocytic cultures are cultured from E17-18 embryos harvested from a Sprague Dawley pregnant female, while chick astrocytic cultures are derived from E14-16 embryos. The cortical regions of the brains collected from these embryos are isolated and their meninges removed. They are then dissociated in serum containing media by trituration. All astrocytic cultures are maintained in 10% fetal bovine serum (FBS from Wisent) in Dulbecco's Modified Eagle's Medium (DMEM from Invitrogen) and cultured on gelatin-coated flasks. Purification of astrocytic cultures is achieved by shaking flasks at 200-250 rpm in DMEM enriched with MEM vitamin supplement (from Invitrogen) overnight. The remaining adherent cells are then 95-99% pure astrocytes since they are 95-99% glial fibrallary acidic protein positive, a protein specific to astrocytes (confirmed by Huashan Peng in the lab). Astrocytes are harvested from the culture flasks by lightly trypsinizing the cells with 0.1% Trypsin/EDTA (Wisent) for 5 min and then quenching the reaction with 10% FBS/DMEM. Astrocytes are centrifuged at 1000 rpm and resuspended in either serum-free media or serum-containing media depending on the objective of the experiment.

2.2 Plasmids and Oligonucleotides

Plasmids used in this study include myc-tagged FRNK (a gift from Dr Tony Parsons) for knockdown of FAK activation, ILK782 and ILK285 (a gift

from Dr St-Arnaud, McGill University) for knockdown of ILK expression and p3.1 Silencer (a gift from Dr St-Arnaud), the backbone for the ILK constructs, was used as a negative control for ILK 782 and ILK 285. FRNK acts as a dominant negative thereby interfering with FAK activation, while the ILK constructs are siRNA snapbacks that tag ILK mRNA for degradation.

To increase the amount of plasmid, plasmids are transformed in DH5α competent cells by first mixing the cells and about 200 ng of plasmid in ice for 30 min, followed by a 2 min incubation at 42°C. The cell/plasmid mixture is then placed on ice for an additional 2 min before being added to LB media. Cells are placed on a shaker at 37°C and allowed to grow for 1h. Transformed cells are then spun down and inoculated on ampicillin containing LB agar plates. Successfully transformed cells are then allowed to grow on the plates for 12-18h. Single colonies are then picked and grown in large LB cultures. Plasmids are then isolated using the Qiagen MidiPlasmid kit. Concentration and purity of plasmid yield is determined using a photospectrometer, while plasmid size is confirmed by gel electrophoresis.

ILK Stealth Oligo siRNAs (ID RSS# 301329-301331) are used to knockdown ILK expression, Block-It Alexa RFP oligonucleotides to test for transfection efficiency and Stealth Universal Control with medium GC content is ordered from Invitrogen. The sequence of RSS# 301329 is ACGCACUCAAUAGCCGUAGUGUAAU, RSS# 301330 is GGACCAGAGCCAAGCUGUAAAGUUU and RSS# 301331 is

CCCACGUGUGUAAGCUCAUGAAGAU. These constructs are used as specified by the manufacturer.

2.3 Cell Adhesion Assays

Glass coverslips are first UV irradiated for more than 15 min and then coated with different proteins/polyamino acids for 45 min or overnight at 37°C depending on the requirement of the experiment. Substrates used were laminin at 20 μg/mL (from Sigma), fibronectin at 10 μg/mL (from BD Biosciences), type 1 collagen from rat tails at 10 μg/mL (from Sigma), 0.1% gelatin in 1x phosphate buffered saline (PBS) or polylysine at 100 μg/mL (from Sigma). Astrocytes are then harvested in serum free media and then applied to these treated glass coverslips in 24 well tissue culture dishes. Astrocytes are then incubated at 37°C and 5% CO_2 for different time periods and are fixed with either 4% paraformaldehyde (PFA) with 4% sucrose or methanol at -20°C for 10 min followed by 3 washes with PBS with the first wash being 10 min in length for the cells to rehydrate after methanol fixation. If PFA fixation was used, astrocytes are then permeabilized with 0.25% Triton-X-100 and washed 3 times with 1x PBS. Coverslips are then blocked with 10% BSA for 1h at RT or 4°C overnight. This is followed by 3 washes with 1x PBS before incubating the coverslips with the primary antibody in 3% bovine serum albumin (BSA) for 1h at RT or 4°C overnight. The primary antibody is then removed and the coverslips are washed 3 times with 1x PBS before the addition of the secondary antibody. The secondary antibody in 3% BSA is applied for 1h at RT and then replaced by DAPI at 0.1

μg/mL: for 10 min at RT. Coverslips are washed 3 times with 1x PBS and then water. They are then mounted on glass slides with SlowFade Gold mounting medium (Invitrogen) and the coverslips sealed with clear nail varnish. Epiflourescence microscopy is then used to visualise the cells.The adhesion assay is used to test the effects of drugs and small molecule inhibitors on centrosomal assembly and maintenance. A drug or small molecule inhibitor may be applied to the astrocytes in suspension before plating on the coverslips to test its effect on centrosome assembly, or directly to the well after astrocytes are adhered to the coverslips to test centrosome maintenance.

2.4 Cell Dissociation Assay

Glass slides are coated with 100 μg/mL polylysine and allowed to dry at 37°C. Astrocytes are then harvested in serum free media and left in suspension at 37°C and 5% CO_2 for different periods of time. Cells are centrifuged at 1000 rpm for 5 min and washed with 1x PBS before resuspended in 4% PFA for 10 min. Astrocytes are then washed and resuspended in 1x PBS, smeared on a polylysine coated glass slide and allowed to dry. 0.25% Triton-X-100 is then applied directly on the glass slide and used to permeabilize the cells for 10 min. Cells adhered to the glass slide is then washed with 1x PBS 3 times. This is followed by 10% BSA to block non-specific antigens for 1h at RT or at 4°C overnight. Primary antibodies are diluted in 3% BSA and applied for 1h at RT or 4°C overnight, then washed 3 times with 1x PBS. Secondary antibodies in 3% BSA are applied for 1h at RT followed by a similar wash. DAPI at 0.1 μg/mL is then added for 10 min

and cells are washed with 1x PBS and water before a small drop of SlowFade Gold is applied. A glass coverslip is then mounted on the slide and sealed with clear nail varnish. Epiflourescence microscopy is then used to visualise the cells.

2.5 siRNA Plasmid and Oligo Transfection

Astrocytes are grown on gelatin coated glass coverslips to 80-90% (plasmid) or 50-60% (oligo) confluency. 1-3 h before transfection, media are changed to serum and antibiotic free DMEM. For each well, 1.5 μL (plasmid) or 1 μL (oligo) Lipofectamine 2000 (from Invitrogen) is incubated in 50 μL serum free media for 5 min. In a separate microfuge tube, 200-400 ng of plasmid or 40-80 nmol of oligos are added to 50 μL of serum free media. The Lipofectamine 2000 and plasmid or oligo mixture are then added together in equal volumes and incubated at RT for 20 min. After this incubation, the mixture is added directly to the well. Cells are harvested 48h or 24h after transfection by fixation with 4% PFA or methanol at -20°C for 10 min. If PFA fixation is used, cells are permeabilized with 0.25% Triton-X-100 for 10 min. Coverslips are then blocked, stained, mounted and visualised as mentioned previously in the adhesion assay.

2.6 Drugs and Small Molecule Inhibitors

PF-228 is a gift from Dr Tony Parson's lab and Pfizer. It is used to inhibit the activation of FAK and is shown to be effective when applied to well-adhered astrocytes at 10 μM for 4 h[191]. Secramine A is a gift from Drs Kirchhausen and Hammond and used to knockdown activation of Cdc42[192]. Secramine A is used at

the comparable concentration of 10 μM for 1-4h as reported previously[192, 193]. Each of these molecules is used to test their effect on the centrosome in attached astrocytes. Astrocytes are allowed to attach to gelatin coated glass coverslips for 4h in 10% FBS containing media. The media is then changed to serum free media containing the appropriate concentrations of each drug or molecule with dimethyl sulphoxide. These cells are allowed to incubate for various lengths of time before cells are fixed by paraformaldehyde or methanol at -20°C. Coverslips are then similarly processed as mentioned in the Adhesion Assay.

2.7 Antibodies

Primary antibodies used are anti-γ-tubulin (monoclonal from Sigma (clone GTU-88: T6657) and polyclonal from AbCam (ab11317), both at 1:500), pericentrin (from Covance (PRB-432C), 1:250), ILK (from AbCam (ab2283), 1:200), FAK397 which specifically tags activated FAK phosphorylated at tyrosine 397 (y-397) (from Invitrogen (44-624G), 1:500), vinculin (from Sigma, 1:100), JG22, a chick specific β1-integrin function blocking antibody, (from Developmental Studies Hybridoma Bank, 1:500 to stain, 1:50 to block function) and β1-integrin antibodies produced in the lab (β1-N, a polyclonal rat specific β1-integrin function blocking antisera produced with the entire β1-integrin subunit, as well as an antiserum to the β1-integrin peptide, both at 1:1000).

Secondary antibodies used are donkey anti-mouse Alexa Flour 488, 555 (from Invitrogen, all at 1:1500) and goat anti-mouse rhodamine (from Jackson Laboratories, 1:1500).

3 Results and Conclusions

3.1 Centrosomes disassemble when astrocytes are in suspension.

If β1-integrin signalling is important for centrosome maintenance, the disengagement of integrins when cells detach from their substrate should cause the disassembly of the centrosome. Upon detachment of astrocytes from their substrate, centrosomes were observed to disassemble rapidly within 1hour (h) (Figure 2A, second panel, and 2B). This disassembly was detected by a failure of γ-tubulin or pericentrin to localize to a distinct focus. Over 4h in suspension, it was noted that both γ-tubulin and pericentrin staining become more diffuse indicating the full disassembly of the centrosome (Figure 2A, rightmost panel). This process seemed to occur relatively quickly, with 70-80% of astrocytes surveyed having no centrosome within 1h of detachment (Figure 2B). By 4h, only approximately 7% of cells still had centrosomes (Figure 2B). Centrosomes that persisted appeared considerably smaller than those at earlier timepoints. From these results, it seems that the detachment of astrocytes from their substrate is sufficient to disassemble centrosomes.

3.2 Centrosomes assemble rapidly on a variety of substrates.

Next, the kinetics of centrosome assembly was studied. Understanding the kinetics of centrosome assembly would allow for a better idea of the time frame that assembly occurs in and the steps the cell undergoes to form an intact centrosome. Astrocytes were maintained in suspension for 2h prior to cell adhesion to ensure disassembly of the centrosome. Centrosomes were detected in

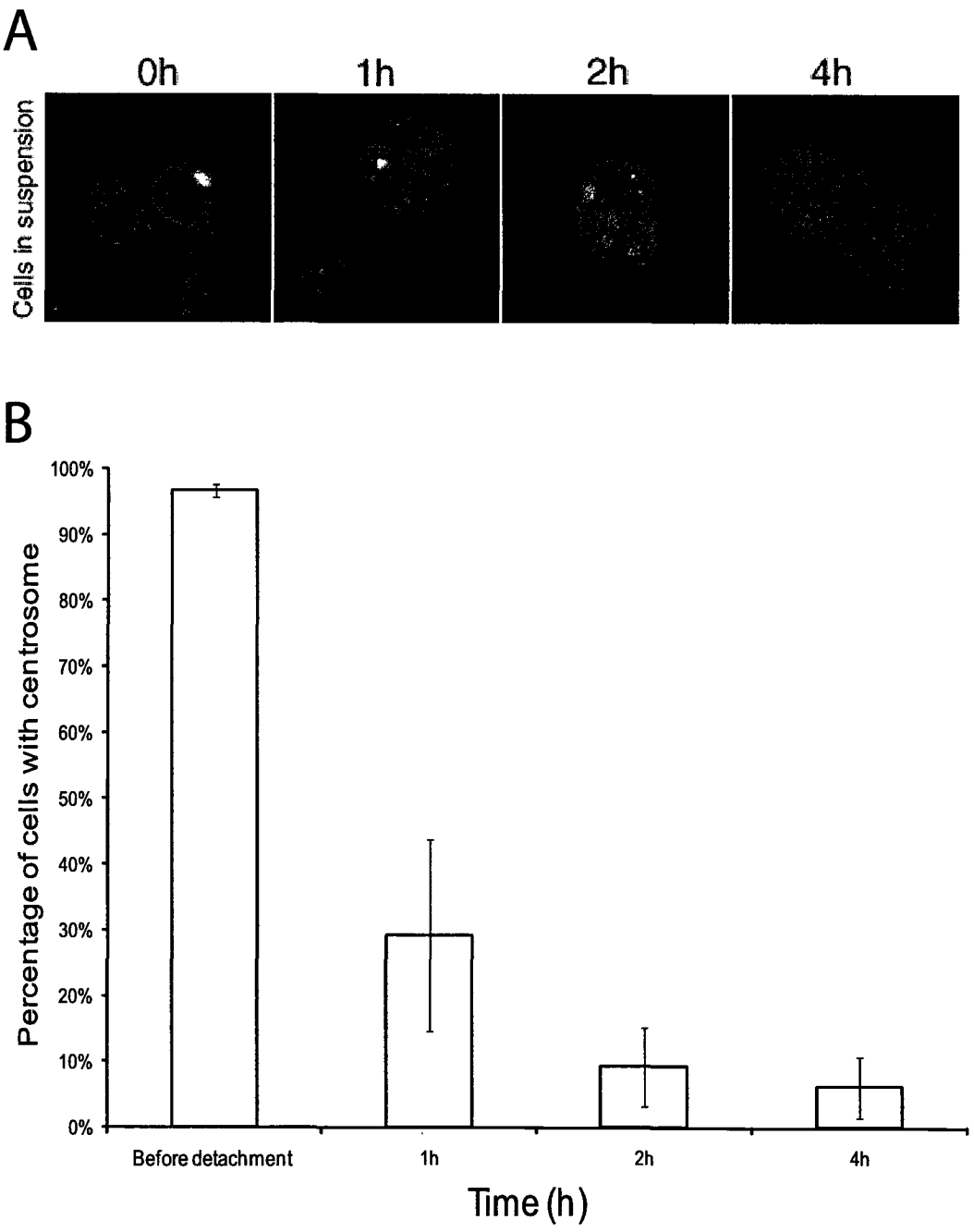

Figure 2: Centrosomes disassemble when astrocytes are in suspension.

Figure 2: Centrosomes disassemble when astrocytes are in suspension.

Upon detachment from the substrate by gentle trypsinization, astrocytes in suspension for up to 4h disassembe their centrosomes (A). Centrosomes were detected with pericentrin (green) and γ-tubulin (red) staining. Before detachment, almost 100% of astrocytes contained an intact centrosome (B). Over time, both pericentrin and γ-tubulin staining became more diffuse within the cytoplasm of the cell while losing their co-localization (A), indicating the disassembly of the centrosome. This disassembly seemed to occur relatively rapidly, with up to 70-80% of astrocytes losing their centrosome within 1h after detachment (B). Astrocytes showed maximal disassembly of centrosome after 2-4h in suspension, with less than 10% of cells containing intact centrosomes (B).

astrocytes adherent to laminin, collagen, fibronectin and polylysine as early as 10min after cells were applied to coated glass coverslips (Figure 3A, leftmost column). Over the course of 4h, cells were observed to spread on each substrate and their centrosomes appeared more focused with a tighter concentration of centrosomal proteins stained adjacent to the nucleus (Figure 3A). Within 10 min, 50-60% of astrocytes had well-formed centrosomes that formed distinct foci (Figure 3B). By 4h, 70-80% of astrocytes observed had obvious centrosomes (Figure 3B). There was very little significant differences in centrosome assembly between the different substrates tested and astrocytes on each substrate assembled centrosomes at similar rates (Figure 3B).

3.3 β1-integrin ligation is necessary for centrosome assembly and maintenance.

In order to test the requirement for β1-integrin ligation in centrosomal assembly, rat astrocytes were incubated in suspension 1 h before being plated on polylysine coated coverslips with the β1-N antibody, a polyclonal antibody that targets β1-integrins at the cell surface and blocks their function. Incubation of cells in suspension was to ensure that β1-integrin signalling is blocked with the antibody and also that astrocytes disassembled their centrosomes before attachment. Cells adhered and spread on polylysine and showed disrupted centrosomes (Figure 4A, top row). Polylysine allowed for the attachment of cells via charge interactions, an integrin independent manner, since the disruption of integrin function disrupts cell adhesion to substrates such as fibronectin and

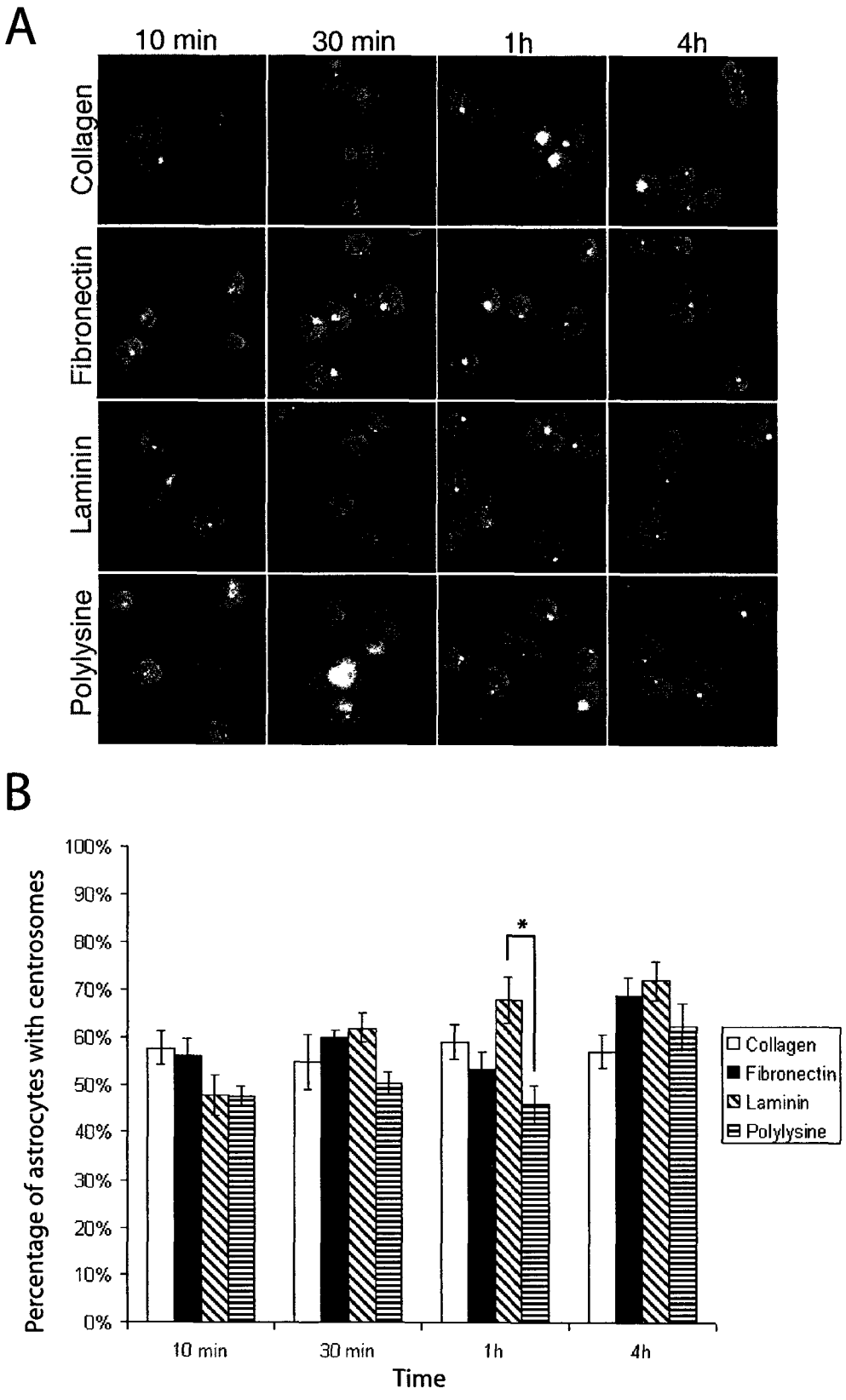

Figure 3: Centrosomes assemble rapidly on a variety of substrates.

Figure 3: Centrosomes assemble rapidly on a variety of substrates.

Astrocytes were in suspension for 2h before being plated in substrate coated coverslips. Centrosomes were identified by staining for pericentrin (green) and were observed to form foci as early as 10 min after attachment on all substrates tested (A) in 50% of the cells observed (B). Over the course of 4h, pericentrin staining accumulated and focused tightly adjacent to the nucleus in astrocytes on all substrates tested (A), with upwards of 70-80% of astrocytes containing well formed centrosomes.. At 10 min, centrosomes appeared as a hazy dot adjacent to the nucleus (A, leftmost column), but over 4h, this hazy dot coalesced to form a tighter and more distinct focus (A, rightmost column). There was very little significant difference observed in astrocytic centrosome assembly among all the substrates tested, collagen, fibronectin, laminin and polylysine (B). The only significant difference was observed at 1h between centrosome assembly observed in cells on laminin and polylysine (B, * $p < 0.01$). This difference was not considered of importance as it was the only difference observed across all time points and substrates tested and was hence disregarded. Although there was no significant difference between different timepoints, we hypothesize that the centrosome is assembling since we reported that the majority of centrosomes disassemble when astrocytes detach from their substrate and are maintained in suspension for 2h, making minimal number of cells containing centrosomes before they are applied to the glass coverslip.

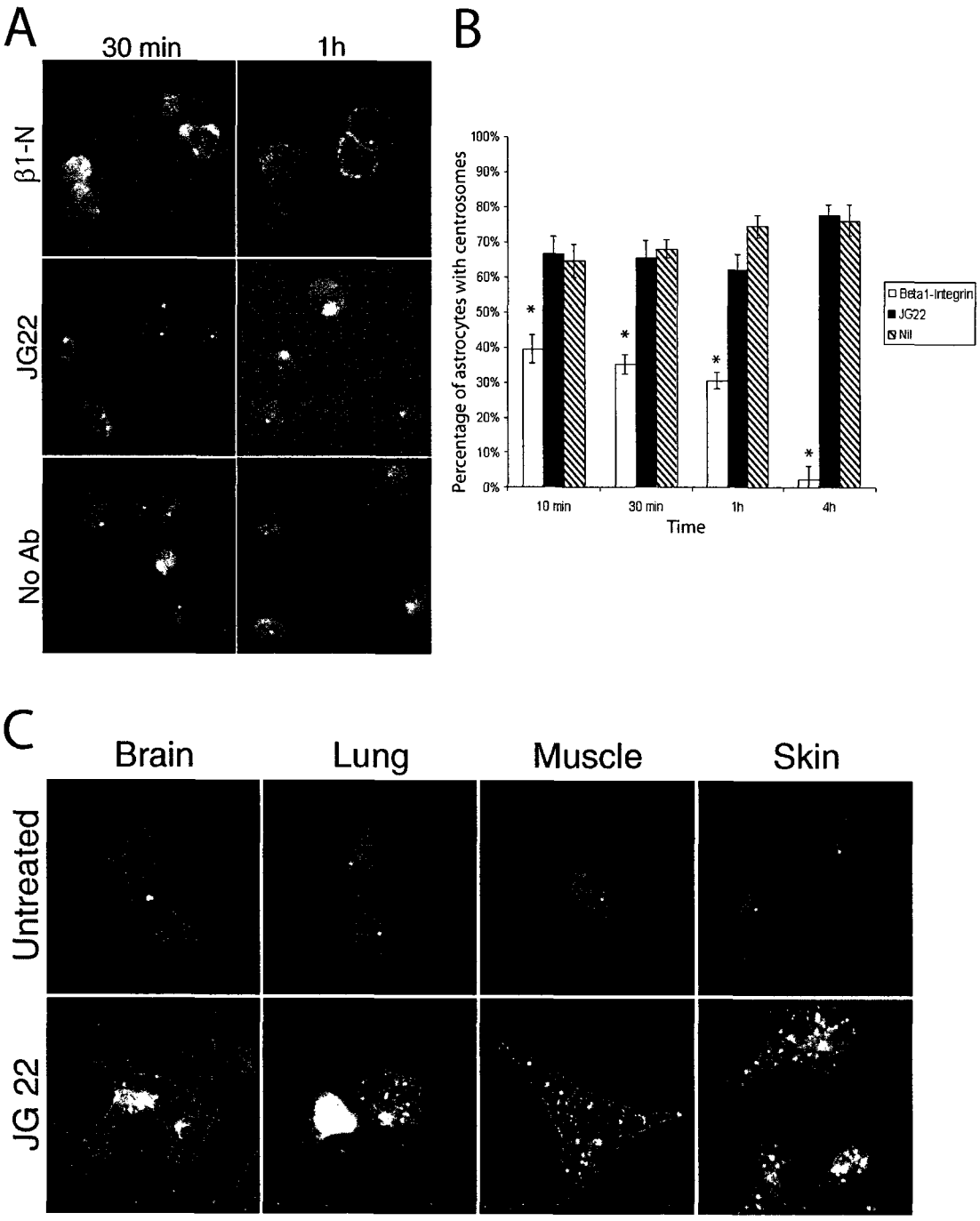

Figure 4: ß1-integrin signalling is necessary for centrosome assembly and maintenance.

Figure 4: β1-integrin ligation is necessary for centrosome assembly and maintenance.

Rat astrocytes were treated with β1-N to block β1-integrin function for 1h before cells were attached to polylysine coated coverslips. Blocking integrin function disrupts cell adhesion to substrates like fibronectin and laminin. Polylysine allows cells to adhere via charge interactions, instead of through an integrin dependent manner. Centrosomes (identified by pericentrin in green) were observed to form at 30 min in control cells treated with JG 22, a chick specific β1-integrin function blocking antibody used as a negative control, or untreated astrocytes (A, bottom two rows), however in cells treated with β1-N, centrosomes did not assemble and in fact continued to disassemble despite the fact that the cell had attached (A, top right panel). Pericentrin staining appeared disrupted compared to the tight foci observed in the controls and, over time, staining was seen in the cell periphery (A). Over time, β1-N treatment caused increasing disruption of centrosome assembly and maintenance (B). Control (JG 22) treated and untreated astrocytes did not show any significant difference in centrosome assembly and was significantly higher than compared to those treated with β1-N (B). Different chick cell types, brain, lung, muscle and skin, were treated with JG 22 to block β1-integrin function and shown to disrupt the integrity of the centrosome (γ-tubulin in green) (C). γ-tubulin staining revealed multiple aggregates of the protein in the cytosol (C, bottom row) compared to the single foci in untreated cells. This experiment showed the requirement of β1-integrin signalling for centrosomal stability independent from cell attachment and regardless of cell type and species.

laminin. Pericentrin staining was diffuse and often occupied a halo in the cell periphery as compared to the tight and distinct foci of pericentrin staining in untreated or JG22 treated cells which showed the centrosome adjacent to the cell nucleus (Figure 4A, top row compared with bottom 2 rows). Disruption of centrosomal integrity was observed as early as 30 min after plating the β1-N treated astrocytes on coverslips with an increasing percentage of cells appearing to lose centrosomal stability over time (Figure 4A, top row, and 4B). Disruption of pericentrin staining initially appeared diffuse just surrounding the nucleus, but over time, migrated into the cell's periphery (Figure 4A, top right panel). Interestingly, the intensity of pericentrin staining also appears to increase over time with centrosomal disruption. This might indicate an upregulation of pericentrin production in response to the inhibition of β1-integrin signalling.

The disruption of centrosome integrity when β1-integrin function is blocked is not restricted to astrocytes. Dissociated cells from chick brain, skin, muscle and lung were attached to polylysine and treated with JG22, a chick specific β1-integrin function blocking antibody. In all tissues sampled, JG22 treated cells showed no change in attachment to their substrate, but showed disrupted centrosomes. γ-tubulin staining appeared to be upregualated, with a higher staining intensity, compared with untreated cultures (Figure 4C). Also, aggregates of γ-tubulin were observed throughout the cell which appeared as supernumerary centrosomes (Figure 4C, bottom row).

These results allow us to conclude that β1-integrin signalling is necessary for proper centrosome assembly and maintenance and that this requirement is not

restricted to astrocytes but is also vital for centrosome maintenance in other cell types, in particular the skin, muscle and lung cells. Having astrocytes well-adhered to polylysine show centrosome disruption with β1-integrin function blocked tells us that adhesion of astrocytes on a substrate is insufficient for centrosomal assembly and that β1-integrin signalling seems critical for centrosomes to assemble and be maintained. Another important implication from this set of experiments is that this pathway seems to be conserved and is important across different species. Both rat and chick cells seem to require β1-integrin signalling for the maintenance of their centrosomes. This conservation between different species would imply that similar mechanisms may be at work in humans as well. Knowing the importance of β1-integrin signalling in centrosome dynamics in humans would give us a better understanding of the mechanism behind human diseases that result from centrosomal dysfunction.

3.4 Astrocyte attachment to different laminin fragments slows centrosomal assembly.

The E3 laminin fragment has been shown to engage dystroglycan[194, 195] and sulfatide[195], but not integrins, while the E8 fragment engages α3β1, α6β1, α7β1 and α6β4 integrins[196-199] (Figure 5A). The integrins engaged by E8 are found predominantly in focal adhesions and participate in well known integrin signalling pathways via FAK and ILK. In an attempt to confirm the necessity of β1-integrin signalling for centrosomal assembly and maintenance, astrocytes were

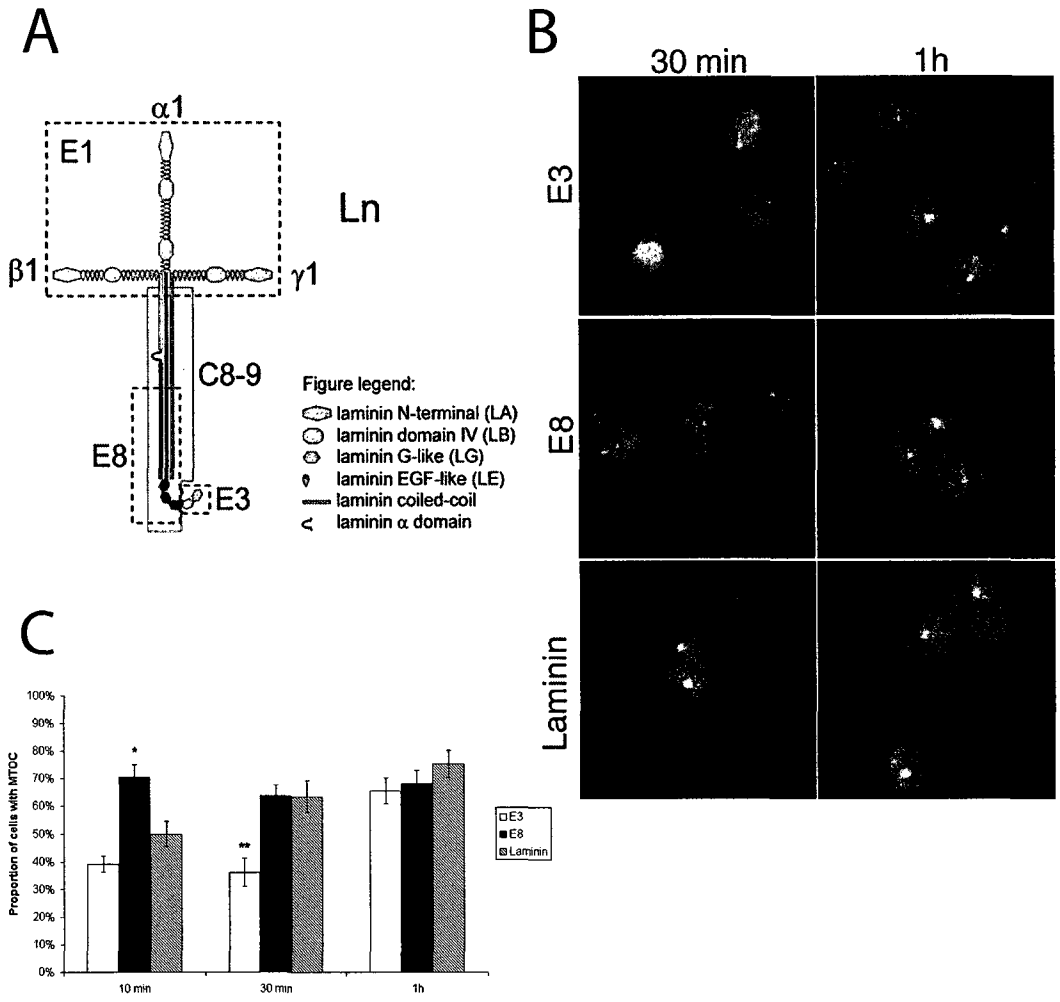

Figure 5: Astrocyte attachment to different laminin fragments slows centrosomal assembly.

Figure 5: Astrocyte attachment to different laminin fragments affects the rate centrosomal assembly.

Rat astrocytes were applied to coverslips coated with the E3 or E8 laminin fragment and centrosome assembly compared to that of astrocytes attached to full length laminin. E3 and E8 are domains within laminin that engage specific receptors, for example E8 is known to bind $\alpha3\beta1$, $\alpha6\beta1$, $\alpha7\beta1$ and $\alpha6\beta4$ integrins and E3 to dystroglycan (A). No significant difference in the rate of centrosome assembly was observed in astrocytes attached to both E8 and full length laminin at 30 min and 1h after attachment (B). However, a slight significant difference (*: $p < 0.05$) in centrosome assembly was noted at 10 min between astrocytes on E8 and full length laminin (B). The difference could be attributed to cells' natural variation in centrosome assembly since no pre-incubation of cells in suspension was carried out before allowing cells to attach to their substrate, the results at this time point were disregarded. This difference in centrosome assembly between astrocytes attached to E8 and laminin was insignificant by 30min (B). Astrocytes attaching to E3 show an initial slower rate of centrosome assembly compared to E8 and full length laminin (B, **: $p < 1\%$, left column, and C). However, assembly of the centrosome on E3 does eventually catch up with E8 and full length laminin after 1h (B, right column, and C).

plated on different laminin fragments, E3, E8 and full length laminin (Figure 5A), and stained for the centrosome with pericentrin. The E8 fragment is only known to engage 4 known integrin heterodimers, this allowed us to observe if these 4 heterodimers, α3β1, α6β1, α7β1 and α6β4, are sufficient to assemble centrosomes. At 30 min after plating, astrocytes adhered to E8 and full length laminin showed a significantly higher percentage of cells that contained distinct centrosomes as compared to that of astrocytes on the E3 laminin fragment, contrasting 70% seen in astrocytes on E8 to 40% observed in those on E3 (Figure 5B and 5C). However, after 1 h of attachment, no significant difference in the percentage of cells containing centrosomes was observed across all substrates tested (Figure 5B) and 4C).

From this, it seems that the E3 fragment while not directly engaging β1-integrins is able to activate the pathways necessary for centrosomal assembly and maintenance. E3 seems to activate these signalling pathways at a slower rate as compared with integrin binding to E8 or full length laminin. These results were surprising since we had previously shown the importance of β1-integrin signalling in centrosomal assembly and maintenance and these results indicate that centrosomes still form even in the absence of direct engagement of β1-integrin, it might be possible that β1-integrins are being activated through an unknown mechanism upon the E3 engagement of dystroglycan. A possibility is that indirect *in cis* interactions between dystroglycan and integrins within the membrane activates integrin signalling[200, 201]. It should also be noted that with the E8 fragment showing results similar to that of full length laminin, it would suggest

that integrins are the primary membrane signalling factor for centrosome assembly and maintenance.

3.5 Activated FAK and ILK localize to focal adhesions in astrocytes on fibronectin and laminin but not polylysine.

With the novel finding that β1-integrin signalling is necessary for centrosomal assembly and maintenance, we next aimed to identify the downstream components in the integrin signalling pathways responsible dictating centrosomal dynamics. With much known about the downstream effectors of integrins, the top two candidates highly activated by integrins, namely FAK and ILK, were chosen to investigate their role in centrosome stability[150, 155, 168].

Astrocytes attached to fibronectin, laminin and polylysine were stained for activated FAK and β1-integrin. After 1 h of attachment, β1-integrin was observed localized to the edges of the cell periphery, at the lamellipodia, consistent with the findings of Tawil *et al.*[136] (data not shown). However, after 4h β1-integrin staining showed clustering and the formation of focal adhesions when cells were attached to fibronectin and laminin (Figure 6A, comparing top left two panels). Similarly, activated FAK staining also showed very prominent localization to focal adhesions and colocalized extensively with β1-integrins in cells adhered to fibronectin and laminin (Figure 6A, left two columns). This colocalization of activated FAK and β1-integrin was not observed in astrocytes plated on polylysine (Figure 6A, rightmost column). In addition, no formation of focal

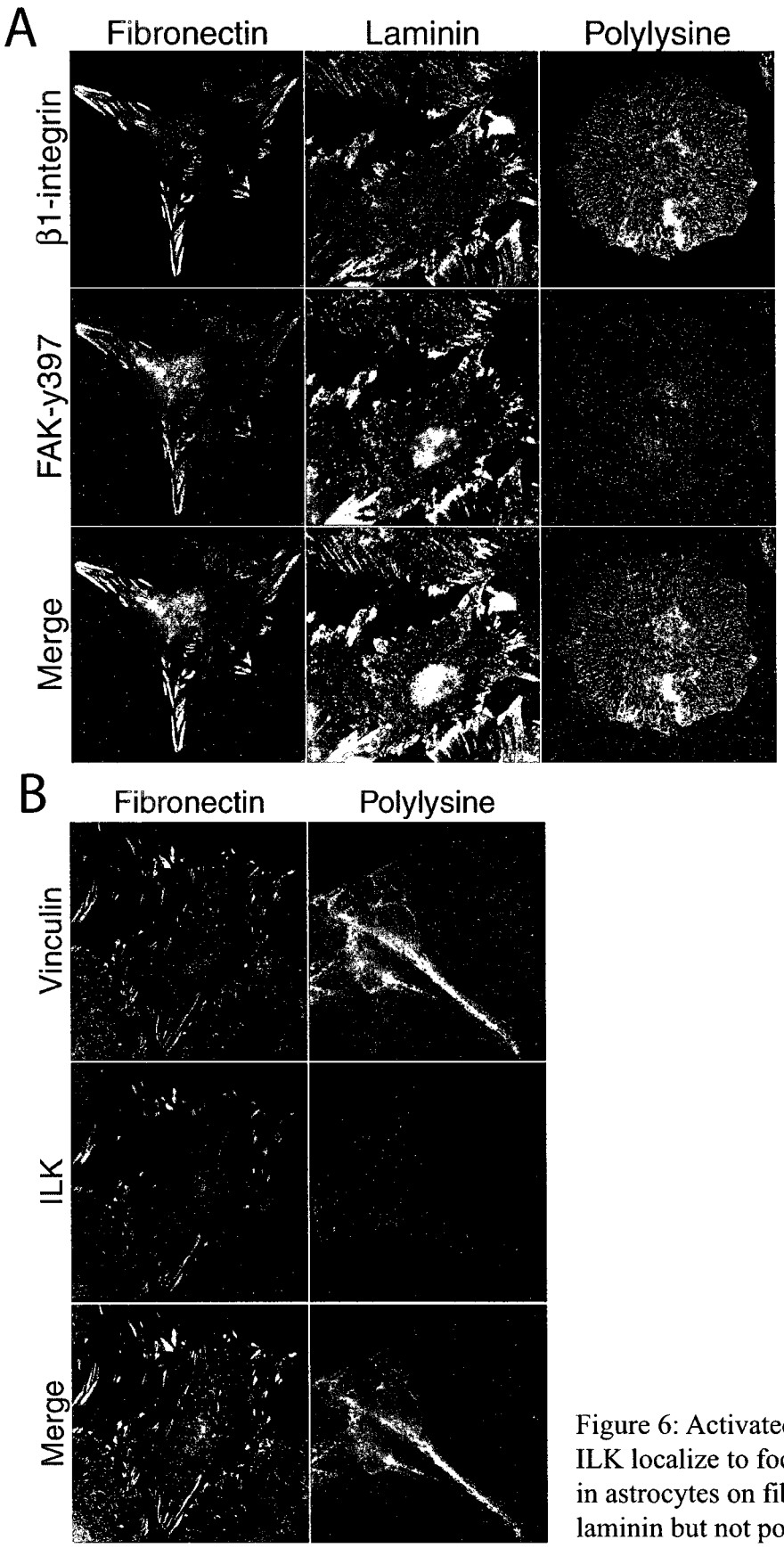

Figure 6: Activated FAK and ILK localize to focal adhesions in astrocytes on fibronectin and laminin but not polylysine.

Figure 6: Activated FAK and ILK localize to focal adhesions in astrocytes on fibronectin and laminin but not polylysine.

Astrocytes were allowed to attach to different substrates for 4h and then stained with FAK-y397, a phosphorylated FAK antibody (Invitrogen), for activated FAK (magenta in A) and β1-integrins (green in A) or ILK (magenta in B) and vinculin (green in B). Astrocytes plated on fibronectin and laminin formed focal adhesions as detected by β1-integrin (A, top left two panels) or vinculin (B, top leftmost panel). β1-integrin could not be used to detect focal adhesion when co-staining with ILK as both are polyclonal antibodies and are not compatible, hence vinculin was used instead. These focal adhesions co-localized excellently with activated FAK (in white in A, bottom row left two panels) or ILK (in white in B, bottom leftmost panel). However, when attached to polylysine, astrocytes do not form focal adhesions and show no co-localization between FAK-y397 and β1-integrin (A, leftmost column) or ILK and vinculin (B, leftmost column). In fact, there appears to be a downregulation of activated FAK (A, middle rightmost panel) and ILK (B, middle rightmost panel).

adhesions was observed in astrocytes attached to polylysine and β1-integrin appeared to be localized to stress fibres throughout the astrocyte (Figure 6A, top rightmost panel). Only a low level of FAK activation was seen and activated FAK did not colocalize with β1-integrin in cells on polylysine and appeared in punctate staining throughout the cell (Figure 6A, right column).

In a similar experiment looking at ILK localization, astrocytes were plated on fibronectin and polylysine. After 4 h of attachment, astrocytes were stained for vinculin and ILK. Vinculin staining labelled focal adhesions in cells plated on fibronectin (Figure 6B, top left panel). ILK showed a similar staining pattern and colocalized extensively with vinculin (Figure 6B, left column). ILK staining showed a punctate staining pattern with no focal adhesion formation, similar to FAK (Figure 6B, right column). In fact, vinculin staining showed no clustering or formation of focal adhesions as well (Figure 6B, top right panel).

Astrocytes attached to fibronectin and laminin engage integrins and initiate the clustering of β1-integrins to form focal adhesions. The formation of these focal adhesions localizes other proteins to form a complex that is responsible for physical attachment and signal transduction. These proteins include vinculin, which serves a structural role in linking the ECM with the actin cytoskeleton and FAK and ILK for the activation of downstream signalling. The formation of these focal adhesions was not evident in astrocytes on polylysine. Furthermore, neither β1-integrins nor vinculin colocalized with FAK or ILK in cells plated on polylysine (Figure 6A and 6B, rightmost columns). Consequently, we were unable to firmly conclude whether canonical signalling pathways like

FAK and ILK were activated on polylysine although this seems unlikely. Although the data seems to suggest that the activation of FAK and ILK on polylysine is unlikely as the staining intensity of both proteins in cells attached to polylysine is markedly reduced (Figure 6A and 6B), there is the possibility that the residual FAK and ILK activity observed in cells plated on polylysine might be sufficient for centrosome assembly. These results indicate that FAK and ILK might not be involved in centrosome regulation.

3.6 Inhibition of FAK activation has no effect on centrosomal integrity.

The first candidate to be studied in the involvement of centrosomal dynamics via integrin was FAK, since FAK is well known to be activated downstream from integrins. FAK activation was reduced by transfecting cells with a dominant negative construct, FRNK[202], using Lipofectamine 2000 (Figure 7B and 7C) or by applying PF-228, a selective small molecule inhibitor of FAK[191] (Figure 7A). Both methods directly disrupt the phosphorylation of tyrosine-397 which is the primary site of phosphorylation of FAK that initiates downstream signalling. Astrocytes attached to fibronectin were treated with PF-228 at 10 μM for 1-4 h in serum free media to well-attached cells. PF-228 inhibits FAK activation, without affecting cell attachment to fibronectin (Figure 7A, red channel). At 1h, astrocytes still exhibited some FAK activation at focal adhesions as well as an undisrupted centrosome (Figure 7A, top row). After 4h of incubation with PF-228, there was virtually no detectable FAK activation anywhere in the cell (Figure 7A, bottom row). This was accompanied with a wholly intact

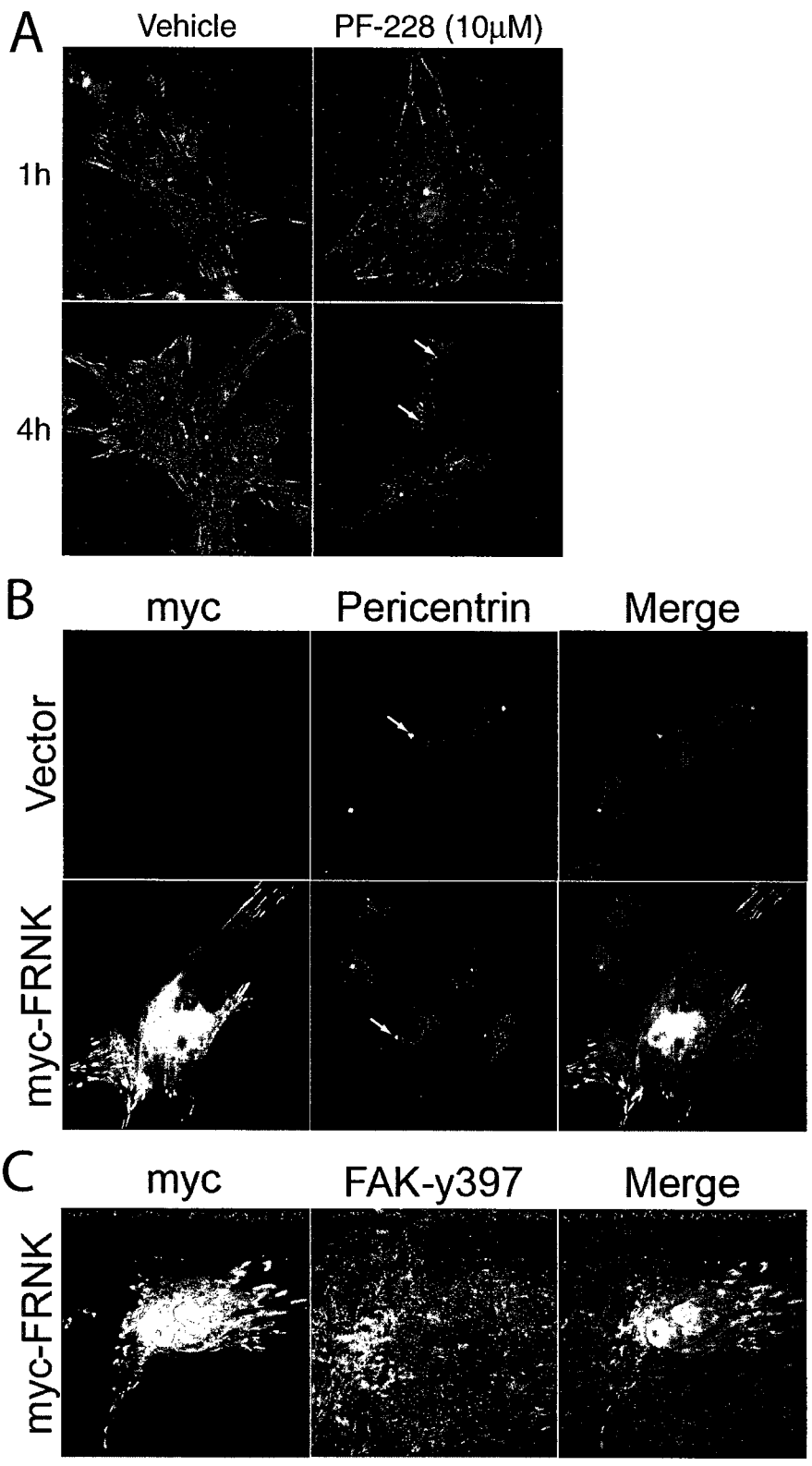

Figure 7: Knocking down FAK activation has no effect on centrosomal integrity.

Figure 7: Knocking down FAK activation has no effect on centrosomal integrity.

FAK activation of rat astrocytes was inhibited by treatment with PF-228 (A), a FAK inhibitor, or transfection with an expression vector encoding FRNK, a dominant negative construct of FAK (B and C). After incubating attached astrocytes with 10 μM PF-228 in serum free media, astrocytes were fixed and their centrosomes and FAK activation visualized. Focal adhesions with activated FAK were still observed after 1h of treatment with 10μM PF-228 (A, top row in red), but extensive knockdown of FAK activation was observed after 4h of treatment with 10μM PF-228 (see loss of FAK-y397 (red) staining (A, bottom row)). Astrocytes that showed a marked loss of FAK activation still showed intact centrosomes compared to their untreated counterparts (A, bottom row, white arrows). In astrocytes transfected with the FRNK expression vector (B, myc-tagged in green), the FRNK protein was localized to focal adhesions (B, bottom rightmost panel in green, and C rightmost panel in green) and showed reduction of FAK activation denoted by decreased staining of FAK-y397 compared to surrounding untransfected cells (C, comparing red staining in green-positive vs. green-negative cells). Astrocytes expressing FRNK also showed no disruption of centrosome integrity and appeared identical to astrocytes transfected with the vector alone (B, white arrows).

centrosome in all the astrocytes surveyed (Figure 7A, bottom row). The reduction of FAK activation by treatment with PF-228 was also documented biochemically. Activated FAK levels were detected by Western Blot analysis and it was revealed that PF-228 did significantly reduce FAK activation by about 95% (unpublished data; Boulos). This indicates that the activation of FAK does not have an essential role in signalling for the maintenance of centrosomal integrity.

To further confirm this conclusion, FRNK was expressed in astrocytes and shown to not disrupt focal adhesions; rather it acts as a dominant negative and inhibits FAK activation[202]. Since the FRNK construct has been myc-tagged, it was visualized with an anti-myc antibody and shown to localize to focal adhesions (Figure 7B, bottom row). Co-staining with activated FAK reflected a reduction in activated FAK levels compared to the vector control (Figure 7C). In 15 transfected cells surveyed by eye, 12 showed marked reduction in FAK activation. However, all transfected astrocytes had normal centrosomes and showed no difference with those observed in untransfected or control transfected astrocytes (Figure 7B, comparing top and bottom rows). Results from these two experiments would eliminate FAK activation from the signalling pathway governing centrosome integrity.

3.7 Knocking down ILK expression has no effect on centrosomal integrity.

The next candidate investigated for its involvement in the maintenance of centrosomal integrity was ILK. Two different types of siRNA constructs were used to knockdown expression of ILK in astrocytes, plasmid constructs courtesy

of Dr St-Arnaud, ILK 285 and ILK 782, and oligonucleotide constructs purchased from Invitrogen. Both types of constructs form short hairpins or double stranded RNAi targeting endogenous ILK mRNA for degradation in the cell. The primary difference between the plasmid construct and the oligonucleotide is that upon transfection, the plasmid construct has to be expressed in the nucleus where the siRNA hairpin is transcribed before it may target ILK mRNA, whereas the oligonucleotide does not require transcription and can act to target ILK mRNA immediately after transfection. Each of these plasmid and oligonucleotide constructs targets different parts of the ILK mRNA, hence using multiple constructs would allow us to confirm any effects observed and be confident that those effects are due to ILK knockdown and not from off-target degradation of functionally related mRNA species.

Positively transfected cells with the plasmid siRNA construct were co-transfected with a marker plasmid and centrosomes were visualized with antibodies against γ-tubulin or pericentrin. ILK localization was also visualized immunohistochemically. Astrocytes that were successfully transfected were identified by the expression of the marker construct, green fluorescent protein (GFP), following plasmid transfection or were assayed for reduced ILK staining and absence of focal adhesions following oligonucleotide transfection (Figure 8A and 8, middle row).

Astrocytes exhibiting GFP, indicating successful plasmid transfections, possessed intact centrosomes compared to control transfected cells (Figure 8B). In addition, the ILK levels within these cells also showed a marked decrease in ILK

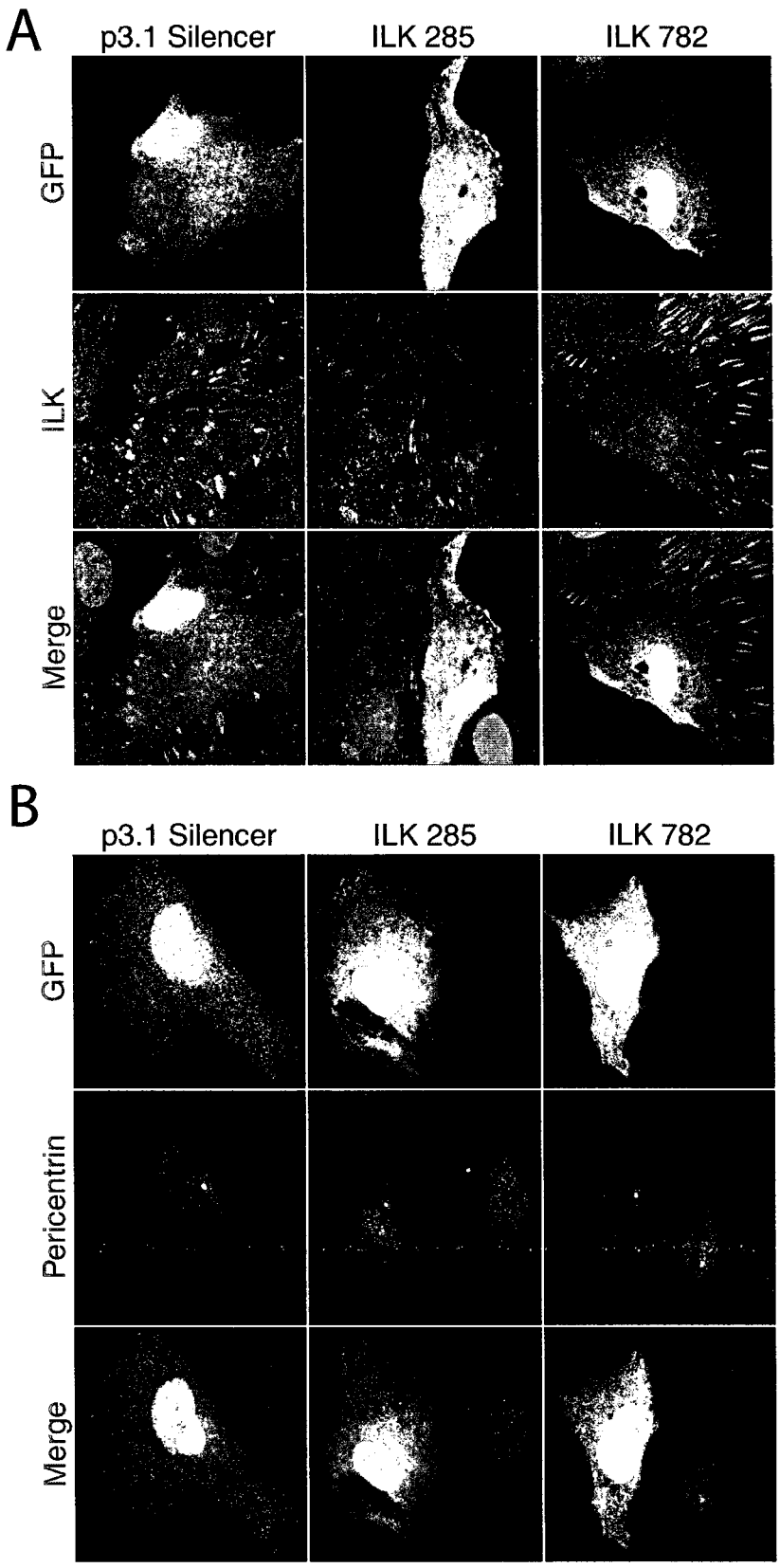

Figure 8: Knocking down ILK expression with ILK siRNA plasmid constructs had no effect on centrosomal integrity.

Figure 8: Knocking down ILK expression with ILK siRNA plasmid constructs has no effect on centrosomal integrity.

Astrocytes were co-transfected with ILK siRNA plasmid constructs, ILK 285 or ILK 782, or with a control plasmid, p3.1 Silencer, and GFP by Lipofectamine 2000 for 48 h. p3.1 Silencer is the backbone plasmid of ILK 285 and ILK 782, without the ILK siRNA construct and hence when expressed in a transfected cell, would produce no siRNA construct. Astrocytes co-transfected with GFP and ILK 285 or ILK 782 showed marked decrease of ILK staining compared to the control (A). When sister cultures of transfected astrocytes were stained to visualise their centrosomes (B, in red and middle row), all cells observed had fully intact centrosomes with no loss in integrity compared to control transfected astrocytes (B). Positively transfected cells could not be stained simultaneously with ILK and the centrosomal marker due to constraints faced with quadruple labelling of the cells and so sister cultures were used to show the effects of ILK knockdown on both ILK expression (A) and the centrosome (B).

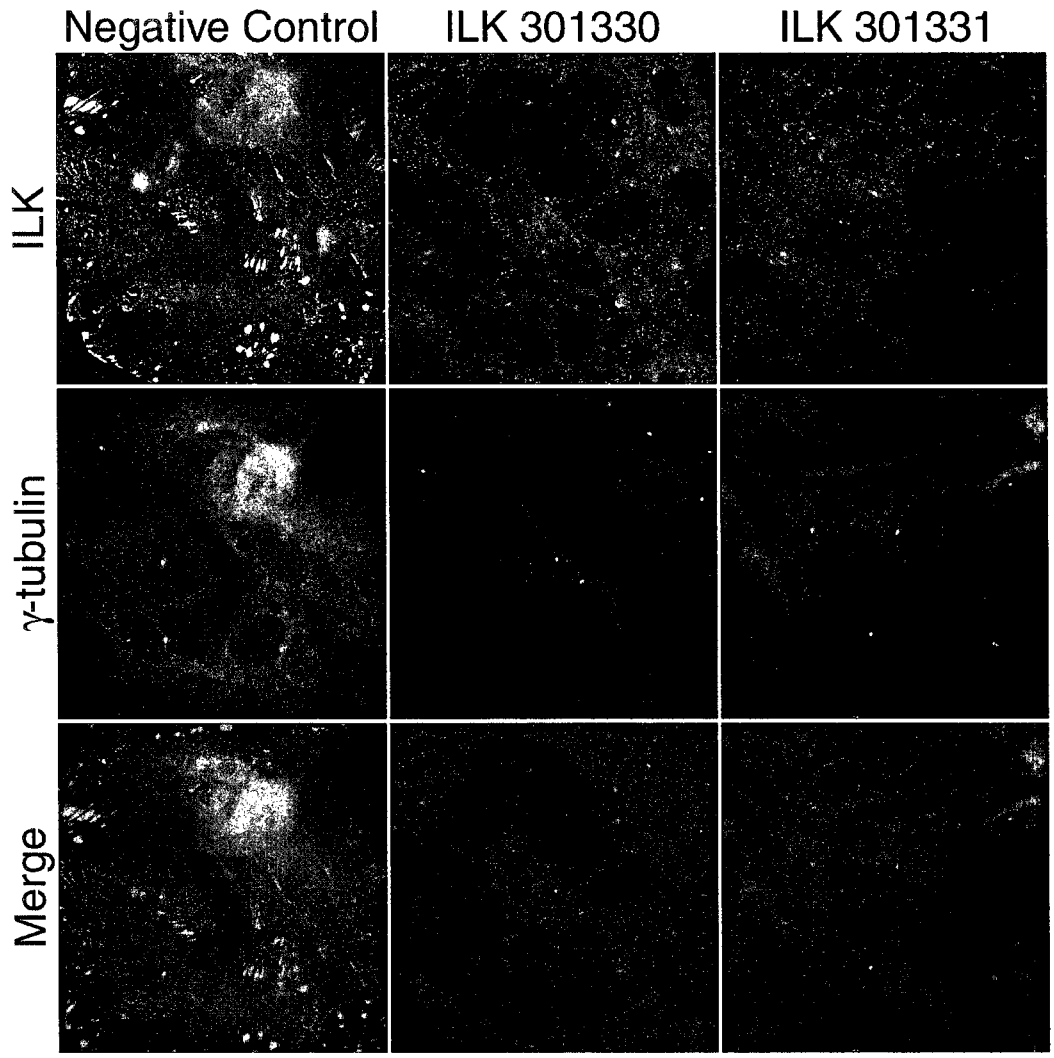

Figure 9: Knocking down ILK expression by ILK oligo siRNAs had no effect on centrosomal integrity.

Figure 9: Knocking down ILK expression by ILK oligonucleotide siRNAs had no effect on centrosomal integrity.

Astrocytes transfected with ILK 301330 and ILK 301331 for 24h showed visible reduction in ILK staining and an absence of focal adhesion in the majority of cells observed (top row). In astrocytes lacking focal adhesions and exhibiting a reduction in ILK staining (top row and bottom row in red), centrosomes did not show any disruption or loss of integrity (middle row and bottom row in green) and looked similar to astrocytes transfected with the negative control siRNA construct (leftmost column). This negative control siRNA construct was purchased from Invitrogen and is a scrambled siRNA construct with similar GC content to that of ILK 301330 and ILK 301331. This negative control siRNA construct has been guaranteed to not contain any off-site targets and ensures that no RNA knockdown occurs when this construct is transfected.

staining intensity compared to the control transfection (Figure 8A). Due to technical constraints of labelling cells with four antibodies, co-staining of ILK and the centrosome was not possible. In order to determine the effect of ILK knockdown on the centrosome, sister cultures were transfected with the ILK siRNA plasmids and stained for either ILK or the centrosome. Control transfection was carried out with the plasmid p3.1 Silencer which is the backbone of the ILK 285 and ILK 782 plasmids without the siRNA construct. A drawback to using p3.1 Silencer as a negative control is that the transfection of this construct alone might be insufficient to trigger the siRNA machinery within a cell which might have non-specific effects. An additional control should be added where astrocytes are transfected with an unrelated gene like GFP and a vector with a scrambled si RNA construct to ensure that there are no additional effects on the cell. With no centrosome disruption apparent in ILK siRNA transfected cells, this implies that ILK is not involved in the signalling pathway that governs centrosomal stability.

In a separate experiment using the ILK siRNA oligonucleotides in transfection, a lack of focal adhesions and reduced ILK staining indicated successful transfection. In addition, positively transfected cells showed no sign of centrosomal disruption and all cells observed possessed intact centrosomes (Figure 9). ILK levels in oligonucleotide siRNA, RSS#301330-301331, transfected astrocytes were visibly reduced (Figure 9, top row). Putting both these results together would indicate that the knockdown of ILK has no bearing

on centrosomal integrity and would imply that ILK is not a candidate in the signalling pathway necessary for centrosomal assembly and maintenance.

3.8 Perturbing Cdc42 activation with Secramine A has no effect on the centrosome.

Another well known downstream target of integrin signalling is Rho-GTPase, Cdc42[59]. This places Cdc42 as a possible candidate in the signalling pathway downstream from β1-integrin that regulates centrosomal stability. Incubating astrocytes with Secramine A would allow us to determine whether Cdc42 is truly in the pathway controlling centrosome maintenance. Previous work by Etienne-Manneville and Hall looked at disrupting Cdc42 function and its effect on cell polarization[78]. Their results did not report any disruption to the centrosome even when the centrosome was used as a marker for cell polarization[78]; knowing this, we would anticipate that Cdc42 is not downstream of β1-integrin in this pathway that regulates the centrosome.

Cells attached to fibronectin and polylysine were then incubated with 20μM Secramine A for 1h. Secramine A is a small molecule inhibitor of Cdc42 and has been shown to reduce its activation by acting as a dominant negative[192, 193]. It has been previously reported that treatment of cells in culture with 20μM Secramine A for 1h selectively inhibits the activation of Cdc42[192]. Treatment with Secramine A also caused no changes in cell-substratum attachment compared with untreated cells and also did not appear to affect cell viability (Figure 10). The inhibition of Cdc42 after attachment might be the reason why no effect was

participate in cell adhesion[203]. Upon further inspection of centrosomes in astrocytes treated with Secramine A, they were intact and well-formed (Figure 10, bottom row in green). However, the direct confirmation that Secramine A causes Cdc42 inhibition in our hands is still pending. With confirmation of Cdc42 inhibition, this would exclude Cdc42 activation from the pathway involved in centrosomal stability and allow the investigation into other pathways that controls centrosomal integrity.

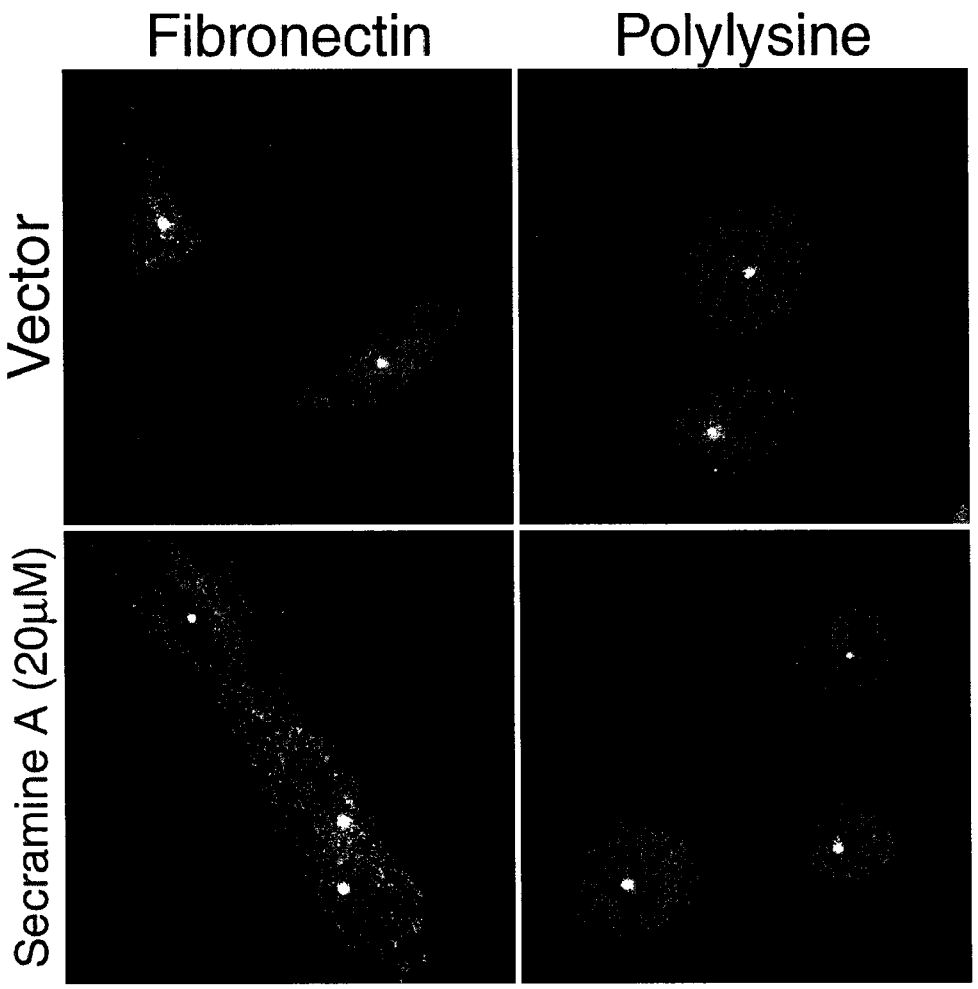

Figure 10: Perturbing Cdc42 activation with Secramine A has no effect on the centrosome.

Figure 10: Perturbing Cdc42 activation with Secramine A has no effect on the centrosome.

Astrocytes attached to fibronectin and polylysine were treated with either the vector (DMSO) or 20μM Secramine A in DMSO. Both control treated and Secramine A treated astrocytes on fibronectin and polylysine showed fully intact centrosomes with no loss of integrity (comparing top row to bottom row).

4 Discussion

4.1 Centrosome Kinetics

From the results attained thus far, we can conclude that centrosomes disassemble when astrocytes detach from their substrate and reassemble rapidly when they reattach. This is consistent with the finding that β1-integrin signalling is important in centrosomal assembly and maintenance since β1-integrin engagement is lost when cells detach and this inactivates the signalling necessary to maintain the centrosome. Upon attachment, β1-integrin is re-engaged to activate β1-integrin signalling and hence the centrosome reassembles.

4.2 Centrosomes Form on Polylysine

An unexpected result from the attachment of astrocytes to polylysine was that centrosome assembly was no different than in cells attached to integrin ligands, fibronectin or laminin. Polylysine is highly positively charged and with cell membranes being negatively charged, cells attach to this substrate through charge interactions alone[204], no cellular receptors are known to be selectively or specifically engaged upon binding to polylysine. Hence, ligand-specific activation of β1-integrins is not expected when astrocytes are attached to polylysine. We are still unable to explain this finding and hypothesize that β1-integrins are being activated by some non-specific mechanism. Even more intriguing is the fact that focal adhesions do not form in astrocytes attached to polylysine, hence the β1-integrin signalling being activated specifically initiates centrosome assembly in a focal adhesion independent manner. Without focal adhesion formation in cells

plated on polylysine, a possible avenue of activation is through point contacts. These smaller adhesive complexes were not investigated and at this time we are unable to determine their presence and activation on polylysine. A signalling pathway that seems to be activated through point contacts, and not focal adhesions, is the RhoA pathway[145], which is another possible signalling cascade to consider. An alternative possibility is through mechanotransduction that will be discussed later.

4.3 Ubiquitous Requirement of β1-integrin Signalling in Centrosome Assembly

β1-integrin signalling seems absolutely critical in the assembly and maintenance of the centrosome since blocking β1-integrin function causes the centrosome to lose its integrity. β1-integrin signalling may in fact be more important in the maintenance of the centrosome rather than initiating its assembly. This may be inferred from the loss of centrosomal integrity in β1-integrin function blocked astrocytes over time. From the data (Figure 3A and 3B), we see 40% of astrocytes containing centrosomes and this declines over time, which might suggest that centrosome are attempting to assemble. However, the loss of β1-integrin signalling prevents the maintenance of centrosomal integrity and hence we observe an increasing disruption in the centrosome over time. Another interesting conclusion that can be drawn from this experiment is that adhesion of astrocytes to a substrate is insufficient for centrosomes to assemble. Even with β1-integrin signalling blocked, astrocytes adhered and spread on polylysine, but still had disrupted centrosomes.

The integrin involvement in centrosomal dynamics also seems to be conserved between rats and chicks and is not limited to a single cell type. This suggests a novel and fundamental importance of β1-integrin signalling in centrosomal maintenance. A better understanding of the signalling pathways that govern the changes in centrosomal behaviour would contribute to our insight on diseases and disorders that involve aberrant centrosomal behaviour. For example, the evidence of supernumerary centrosomes in many cancer cells[205] would suggest that perturbation of this pathway might contribute to the anomalies seen in cancer cells. Malignant cells exhibiting supernumerary centrosomes are observed to proliferate by coalescing their multiple centrosomes to form a bipolar spindle during cell division thus ensuring proper segregation of chromosomes and maintaining the genomic stability required for cell survival[190]. Hence, this signalling pathway might become a potential target for the development of therapeutics for cancer treatment.

4.4 Laminin Fragments and Centrosome Assembly

However, the requirement of β1-integrin signalling in centrosome assembly and maintenance is further complicated with the results from astrocytes attaching to different laminin fragments. Laminin fragment, E3, is not known to engage any integrins or activate integrin signalling cascades, rather it is known to engage Dg and sulfatide[195], other cell membrane receptors. Hence, cells attached to E3 were expected to similarly show disrupted centrosomes since β1-integrin signalling activation was not expected. However, centrosomes did indeed form,

albeit at a slower rate as compared to astrocytes attached to E8 and full length laminin. This might be due to *in cis* interactions between Dg and β1-integrins[201] that activate downstream signalling required for proper centrosomal assembly and maintenance. Such interactions would explain the delay in centrosomal assembly since β1-integrins are not activated directly, but via dystroglycan engagement. Another plausible explanation is that laminin might be present on the cell surface which is sufficient for β1-integrin engagement and activation. Presence of laminin molecules can be explained by carry over of laminin when cells are detached.

To determine whether dystroglycan is indeed activating β1-integrins *in cis* and hence centrosome assembly, we may inhibit dystroglycan function by a Dg function blocking antibody, IIH6, in astrocytes and on polylysine, allowing for cell attachment, to see if centrosomes assemble. If Dg is in fact activating β1-integrins through an *in cis* interaction, no assembly of centrosomes should be expected. As for the possibility of laminin molecules being retained on the cell surface during cell detachment, we may test this hypothesis by treating cells with a laminin blocking antibody before attachment. This would bind to laminin molecules on the cell surface and prevent its engagement of β1-integrins. Without this indirect engagement of β1-integrins, the loss of centrosome assembly would be expected when cells attach to the E3 laminin fragment.

4.5 Mechanotransduction of Integrin Signalling

The results thus far reveal a somewhat confusing picture of how β1-integrin signalling is involved in centrosomal assembly and maintenance. There is

an apparent requirement of β1-integrin signalling in centrosome stability (Section 3.3) and yet instances where β1-integrin signalling was not expected, i.e. during cell attachment on polylysine or E3 laminin fragment, centrosomes formed. This begs the question of whether β1-integrin signalling is activated without the binding of specific ligands. This is where the idea of mechanotransduction might possibly fill the gap and explain what has been observed. Reports have shown that during ligand-binding cell adhesion, forces at the cell surface membrane are generated[206]. The phospholipid bilayer undergoes deformation when it comes into contact with its substrates and the cell starts to spread upon adhesion. It has been shown that this deformation produces forces that have been implicated in integrin clustering and activation of its downstream signalling[207]. We hypothesize that similar forces may be present when the cell surface comes into contact with polylysine and initial charge interactions attract the phospholipid bilayer and deforms it. It should be noted that the activation and possibly the conformational changes of integrins is still required for this signalling pathway to be activated and would explain the effects observed on the centrosome when integrin signalling is blocked.

A consequence of force generation during cell adhesion is that force-sensitive proteins which reside within the membrane would get activated and this may in turn initiate other downstream signalling pathways. A force sensing protein that has been discovered is p130Cas[208], a cytoskeletal tyrosine kinase that is also highly featured in focal adhesions. P130Cas has been shown to activate Rap1[208], a small GTPase, which is also known to activate integrin signalling via

Rap1-GTP interacting adaptor molecule (RIAM)[209]. RIAM in turn has been reported to bind to talin and conformationally activate integrin signalling[209]. We speculate that the forces generated during cell adhesion on polylysine are sufficient to activate p130Cas which results in eventual integrin signalling initiation through Rap1 activation. This would explain the assembly of the centrosome on polylysine and its requirement of β1-integrin signalling for its assembly. It would be interesting to investigate whether this mechanism is responsible for the engagement of integrin signalling and results in centrosomal assembly. We might be able to achieve this by looking at the effect of knocking out p130Cas or Rap1 on centrosomal assembly and maintenance.

4.6 Downstream Signalling from β1-integrin

4.6.1 FAK and ILK

In an attempt to identify the direct downstream component of β1-integrin signalling that is involved in centrosomal assembly and maintenance, the first candidates to be considered were the integrin-proximal proteins that are most well known in integrin signalling, namely FAK and ILK (Figure 11). To show their involvement, we first tried knocking down activation or expression of these molecules and observed the effect on the centrosome. However, even with the employment of different methods to knock down both activation of FAK, through dominant negative constructs and small molecule inhibitors, and ILK expression, through siRNAs, no disruption to the centrosome was observed. This implies that the signalling pathway governing centrosomal dynamics might be one that is less

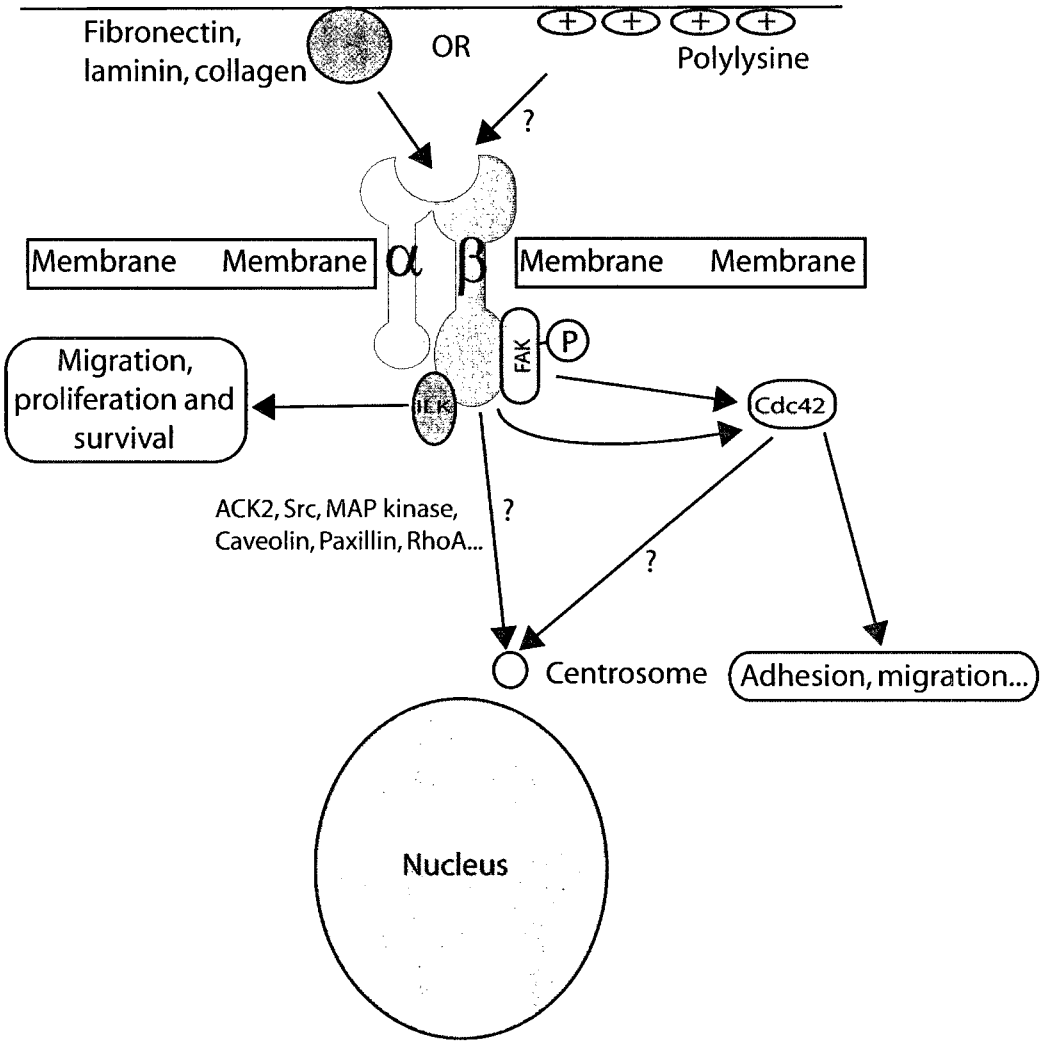

Figure 11: Model for integrin signalling in centrosome assembly and maintenance

Figure 11: Model for integrin signalling in centrosome assembly and maintenance

Integrin ligation with ECM proteins like fibronectin, laminin and collagen or seemingly with polylysine via an unknown mechanism is necessary for centrosome assembly and maintenance. We hypothesize that β1-integrin signalling is responsible for the initiation of the centrosome and its stability and attempt to determine the components of the signalling cascade downstream from β1-integrin. The two candidates considered here are FAK and ILK which are prominent proteins in adhesion, migration, proliferation and survival and well known interactors of β1-integrin. However, our results strongly suggest that FAK and ILK are not involved in centrosome assembly and maintenance. Cdc42 was also a possible candidate and when tested did not appear to be involved in centrosomal stability, although testing was not as rigourous. Current work is still underway, attempting to identify the downstream factor from β1-integrin that is responsible for orchestrating centrsome assembly and its maintenance.

well known. These findings, while unexpected, are consistent with the results we see from the experiments looking at FAK activation and ILK localization in astrocytes attached to polylysine. A reduction in FAK activation and ILK localization was observed in these cells, with the failure of these factors to aggregate to focal adhesions. It is believed that the clustering of these molecules when focal adhesions form is necessary for the activation of FAK and ILK. Hence, without clustering, we do not expect these components to be activated on astrocytes attached to polylysine and yet, centrosomes form normally. Although, we still need to investigate the possibility that these signalling pathways are being activated via point contacts. This might suggest that FAK and ILK are not strong candidates to transduce the signal for centrosome assembly from β1-integrin (Figure 11).

It should be noted, however, that the knockdown of FAK activation in these experiments affected phosphorylation at site 397 only. There are multiple phosphorylation sites on FAK and while site 397 has been shown to be the primary site for activation, the site that is required for controlling centrosomal dynamics might differ[151]. This is provided that FAK need be phosphorylated at all for regulating the centrosome. The presence of FAK might be sufficient for governing centrosome stability and it would be informative to knockdown expression of FAK via siRNAs or the introduction of mutations.

4.6.2 Cdc42

In astrocytes treated with Secramine A, centrosomes were observed to be well formed and intact. Etienne-Manneville *et. al.* showed that perturbation of Cdc42 disrupts reorientation of the centrosome, but did not report any disruption of the centrosome itself[78]. This would strongly imply that Cdc42 is not involved in maintaining centrosomal integrity which is what was observed from our experiment (Figure 11). Although the conditions employed in this study were identical to those reported showing effective knockdown of Cdc42 activation, confirmation of actual disruption of Cdc42 activation still needs to be made.

4.6.3 ACK2

Further exploration for possible candidates has opened a door to a number of possibilities. These include ACK2 which has been shown to be activated in NIH3T3 cells upon adhesion to polylysine[144]. In fact, ACK2 has been shown to be activated without cell spreading, this might indicate early activation at initial contact of the cell with the substrate and would fit the centrosome assembly observed 10 min after cells were plated. ACK2 has also been shown to initiate filopodia formation which is important in cell adhesion and migration, as well as activating JNK1 which is highly involved in cell proliferation and apoptosis.

4.6.4 RhoA

Another candidate is RhoA signalling which is observed at point contacts. Although focal adhesions are not observed to be formed in astrocytes on

polylysine, we have not excluded the possibility that point contacts form. In fact, RhoA has been shown to be suppressed by FAK[210] but is activated in point contacts[145]. Since point contacts form upon initial contact of the cell with its substrate, point contact signalling might be switched on during early attachment[136]. Point contact activation of RhoA plays an important role in axonal guidance and the control of the actin cytoskeletal dynamics involved. Since, the centrosome also plays a role in changes in cell shape and the organization of MTs within a cell, RhoA would be an intuitive candidate to consider downstream from β1-integrin for centrosomal stability. RhoA signalling may be disrupted by a small molecule inhibitor, T-27632[211], to test its effect on centrosomal stability.

4.6.5 Caveolin-1 and Paxillin

Caveolin-1 is yet another molecule that is highly associated with β1-integrin and participates in signal transduction[212]. Caveolin-1 is known to associate with the phospholipids bilayer to form caveolae, invaginations in the cell membrane[213]. This is important for endocytosis and membrane transport[213]. Caveolin-1 has also been shown to interact with integrins in focal adhesions and is believed to initiate specific downstream pathways upon integrin engagement[212]. This ability for signal transduction from β1-integrin would make caveolin-1 a good prospect for study in the process of centrosomal assembly and maintenance. Similarly, paxillin is also associated and activated by integrin activation[141] and should also be included for consideration as a component of the signalling pathway in centrosomal dynamics.

4.6.6 Src Family Kinases

An interesting group of kinases that should be taken into consideration is that of the Src family of kinases (SFKs). SFKs are known to be directly activated by integrins[214]. This gives a candidate that is activated in parallel to both FAK and ILK and would be a good potential signalling pathway that regulates centrosomal stability. SFKs are well known oncogenes that control cell proliferation, motility, differentiation and adhesion as reviewed by Dehm and Bonham[215]. SFK activation has been documented in numerous cancer lines[215]. Their role in proliferation might belie a possible role in controlling the centrosome.

4.6.7 MAP Kinase

Finally, the MAP kinase pathway should be considered. Studies have shown that MAP kinase signalling can be activated with and without FAK activation[216, 217]. The precise position of MAP kinase in the FAK independent pathway is still unknown but it provides an additional contender for controlling centrosomal dynamics without FAK activation. MAP kinase signalling may be downregulated by administering different small molecules like Arctigenin[218] to knock out activation of different components in the pathway. However, with this particular pathway controlling many different cell behaviours, any effect seen on centrosomal integrity would be confounded with the disruption of possibly unrelated factors. We would have to draw conclusions about such experiments with caution. Also, the possible candidates mentioned represents only a small subset of the signalling cascades that are involved with β1-integrin. While integrin

signalling research is vast, we have narrowed down a few candidates to pursue in the near future. Once all these avenues are exhausted, we intend to employ a broader, less specific method to identify signalling molecules downstream from β1-integrins that are also involved in centrosomal dynamics. The usage of RNAi libraries and broad spectrum inhibitors might give insight into candidates that were otherwise excluded from the list before. This allows for novel molecules to be discovered downstream from β1-integrins.

4.7 Current and Future Work

Current work in the lab is investigating the requirement of β1-integrin signalling *in vivo*. Using the embryonic chick retina, attempts have been made to disrupt β1-integrin function with JG 22 and observe the behaviour of the centrosome under such conditions. We expect to observe disruption to the centrosome in tissue treated with JG 22. However, due to technical inconsistencies, we were unable to confirm this conclusion at the time of submission. Showing the importance of β1-integrin signalling in centrosome stability *in vivo* would further validate the importance of this signalling pathway in centrosome dynamics.

Other work currently in progress in the lab is exploring whether integrin signalling without adhesion is sufficient to assemble the centrosome. This is being achieved by activating integrin in cells that are maintained in suspension with integrin-activating antibodies or peptides. If cell adhesion is not required for centrosome assembly, then integrin-activated cells in suspension would contain an

intact centrosome. This result would enable us to separate the requirement of integrin signalling in centrosome assembly and maintenance from that of integrin-dependent cell adhesion.

The two main questions that remain from this body of research are the unknown mechanism by which β1-integrin is activated on polylysine and the downstream signalling cascade that β1-integrin works to control centrosomal behaviour. We hypothesize that the mechanism at work for β1-integrin activation on polylysine, where no apparent integrin is engaged, may occur via p130Cas/Rap1 which detects membrane forces that are generated during adhesion[208]. This is assuming that the deformation of the cell membrane during initial attachment generates forces large enough to be detected by p130Cas. This would explain why astrocytes attached and spread on polylysine still show centrosomal disruption when β1-integrin signalling is blocked. Another possibility is that while focal adhesions do not form on polylysine, point contacts do. These point contacts will contribute to the activation of different signalling pathways upon attachment, with one of these pathways possibly contributing to centrosomal maintenance and stability. Although many signalling pathways are activated by focal contacts and point contacts alike, a more thorough look at the signalling pathways exclusive to point contacts might reveal more interesting contenders for participation in centrosomal dynamics. This leads us to the second major issue which is deciphering the specific cascade β1-integrin employs to exert its effect on the centrosome. An excellent prospect would be RhoA signalling which appears to be activated at point contacts and not focal adhesions.

In an attempt to identify this pathway, we have essentially ruled out the two major contenders, FAK and ILK, as being involved. To reinforce their lack of involvement, we should next attempt to show that overexpression of these proteins would similarly have no effect on centrosomal integrity. This would show that both the inhibition and hyperactivation of both molecules have no effect on the centrosome in effect disallowing the possibility that the effect observed with β1-integrin inhibition is a result of an imbalance of signal activation. This elimination of FAK and ILK as candidates, while unexpected, shows that a possibly novel pathway might be at work and more effort would have to be put in to elucidate the exact signalling pathway in question. Here, we have discussed other possible candidates that we plan to investigate in the future to determine if they have a role in dictating centrosomal behaviour.

Understanding these pathways would allow us a better appreciation of the processes and molecules necessary for controlling the behaviour of the centrosome. The centrosome is an integral organelle to the cell and controls many of the cell's behaviours like cell division, migration, polarization and adhesion as discussed here. Knowing what controls the centrosomal dynamics would enable us to better understand the processes that govern a cell's behaviour and how cells perform their many functions. In addition, with this insight into the mechanism that directs centrosomal dynamics, diseases which involve disruption to the centrosome would be better understood and potential avenues for therapy or treatments may be developed targeting these pathways.

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