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Role of intermediate filaments in mechanotransduction

Gaëlle Dutour Provenzano

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Sorbonne Université and Pasteur-Paris University
International Doctoral Program

Ecole Doctorale Complexité du Vivant

Institut Pasteur, Cell polarity, Migration and Cancer Unit

Doctoral thesis

**Role of intermediate filaments in
mechanotransduction**

Presented by

Gaëlle Dutour Provenzano

Under the supervision of Dr. Sandrine Etienne-Manneville

On the 2nd of November 2021

Before a jury composed of:

Referees: Dr. Sabrina Pichon-Batonnet

Dr. Nicolas Borghi

Examiner: Dr. Catherine Coirault

Guest: Dr. Pascal Silberzan

Supervisor: Dr. Sandrine Etienne-Manneville

“The only way of discovering the limits of the possible
is to venture a little way past them
into the impossible.”
Arthur C. Clarke

To my family,

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Preface

The astrocytes are the guardians of the homeostasis of the brain: with brain lesions after a stroke, an infection or tumour development, astrocytes are requisitioned at the site of the disruption to begin the healing process. There, they will encounter changes in the physical and chemical properties of the Extracellular matrix characterised p.e. by an increase in stiffness and will need to adapt to the differences in the substrate. It is therefore important to describe how the cell changes its morphology and adapt to this new microenvironment. The cytoskeleton of the cell is one of the major actors in the sensing of the extracellular matrix changes, and intermediate filaments having extremely interesting mechanical characteristics, we focus this work on understanding how intermediate filament play a role in the mechanism by which the cell senses and react to new environments.

Intermediate filaments expression is increased during astrogliosis, especially GFAP and also in glioblastoma, the most severe type of brain cancer. Understanding the mechanical pathways used by IFs in a healthy system where cancerous cells derived from, to sense and act on its surrounding environment is crucial to understand how these proteins help the propagation of the disease phenotype, and help find new targets for therapy.

The focus of this project is to determine the role of intermediate filament in the mechanotransduction of glial cells. In part I, we will discuss the general structure and properties of the cytoskeleton, with an emphasis on Intermediate Filaments present in astrocytes. We will carry on with the mechanical properties of the cytoskeleton, to understand better their role in cells. We will then present mechanotransduction and how the mechanical properties of the cytoskeleton are essential in this pathway. We will finally discuss mechanotransduction at the nucleus and how the cytoskeleton mediates nuclear changes. In part V, the objectives of my doctoral research are explained. Part VI consists of the results obtained during the PhD, they will be discussed and interpreted in the following part.

Summary

Cells continuously adapt to their microenvironment. In particular, they modulate their morphology, growth, division, and motility according to the biochemical and physical properties of the extracellular matrix (ECM). Cells are equipped with adhesive structures called FAs, allowing them to interact with ECM proteins through the core transmembrane proteins called integrins and to sense the nature and the rigidity of the ECM. This information are transduced by FA proteins and lead, for instance, to changes in acto-myosin-mediated mechanical tension. Downstream signalling pathways also reach the nucleus; gene expression is then modified and may, in return, affect the composition of FAs or of the ECM proteins for adaptative cell response.

Here, we hypothesized that, in addition to signalling pathways, a direct mechanical coupling between the events occurring at the cell periphery and the nucleus may participate in the transmission of mechanical cues and the regulation of nuclear functions. Although intermediate filaments (IFs) have extremely interesting mechanical properties and resist high tension load, their involvement in mechanotransduction pathways remains elusive. Using astrocyte as a model, due to its specific combination of IFs: vimentin, GFAP, nestin, and synemin, we studied first the effect of substrate rigidity on the nucleus morphology and function, and on the organisation of IFs around the nucleus. Then, we investigated the role of IFs in rigidity-induced nuclear changes. Using a combination of microfabrication techniques, biochemical and microscopy methods, we showed that substrate rigidity affects the nucleus shape, volume, and structure of the chromatin and the recruitment of transcription factor (YAP) and IFs are mediating these changes. Our results suggest that IFs form a cage-like structure around the nucleus in a rigidity-dependent manner: stiffer substrates promote the formation of a cage of vimentin and nestin. In the absence of IFs, the nuclear changes induced by rigidity are different than with IF. The nucleus increases its size in soft substrate, together with an increase in tension measured by YAP localising in the nucleus. The structure of the chromatin is changed. Altogether, the results obtained during our investigation give a better understanding of the role of intermediate filaments in the mechanosensitive nuclear responses.

Résumé

Les cellules s'adaptent en permanence à leur microenvironnement. En particulier, elles modifient leur morphologie, leur croissance, leur division et leur motilité en fonction des propriétés biochimiques et physiques de la matrice extracellulaire (MEC). Elles sont équipées de structures adhésives appelées plaques d'adhérences, permettant aux cellules d'interagir avec les protéines de la MEC via les protéines transmembranaires appelées intégrines et de détecter la nature et la rigidité de la MEC. Le signal est transduit par les protéines des plaques d'adhérences et résulte par exemple en une modification de la tension mécanique induite par l'acto-myosine. Les voies de signalisation en aval peuvent également atteindre le noyau. L'expression des gènes peut alors être modifiée, ce qui peut en retour affecter la composition des plaques d'adhérences et de la MEC pour une réponse cellulaire adaptative.

Nous avons émis l'hypothèse qu'en plus des voies de signalisation, un couplage mécanique direct entre les événements se produisant à la périphérie de la cellule et le noyau pourrait participer à la transmission de signaux mécaniques. Bien que les filaments intermédiaires (FIs) aient des propriétés mécaniques extrêmement intéressantes et résistent à des charges de tension élevées, leur implication dans les voies de mécanotransduction est encore mal connue. En utilisant l'astrocyte comme modèle, en raison de sa combinaison spécifique de FIs : vimentine, GFAP, nestine et synémine, nous avons d'abord étudié l'effet de la rigidité du substrat sur la morphologie, la structure et les fonctions du noyau, ainsi que sur l'organisation des FIs autour du noyau. Nous avons ensuite étudié l'impact de l'absence de FI les changements nucléaires observés en réponse à la rigidité du substrat. En utilisant une combinaison de techniques de microfabrication, de méthodes biochimiques et de microscopie, nous avons montré que la rigidité du substrat affecte la forme, le volume du noyau, la structure de la chromatine et le recrutement des facteurs de transcriptions (YAP). Nos résultats suggèrent que les FI forment une structure en forme de cage autour du noyau d'une manière dépendante de la rigidité : un substrat plus rigide induit la formation d'une cage de vimentine et de nestine. Cette interaction avec le noyau pourrait expliquer les modifications nucléaires observées en réponse à la rigidité du substrat. Au total, les résultats obtenus au cours de notre étude permettent de mieux comprendre le rôle des filaments intermédiaires dans les réponses nucléaires aux propriétés mécaniques du substrat.

Abbreviations

cIFs : cytoplasmic Intermediate Filament

CNS:Central Nervous System

FA: Focal adhesion

GBM: Glioblastoma Multiform

GFAP: Glial Fibrillary Acidic Protein

HDAC6 : Histone Deacetylase 6

IF : Intermediate filaments

MEC-ECM : Matrice Extra Cellulaire; Extracellular matrix

MT : Microtubule

MTOC : Microtubule Organisation Centre

PAP: Presynaptic process

PKA: Protein Kinase A

PTM: Post translational modification

ROS: Reactive Oxygen Species

SIRT2: NAD-dependent deacetylase sirtuin type 2

TNF: Tumour necrosis factor

ULF: Unit length Filament

YAP: Yes Associated Protein

A-TAT : alpha-tubulin acetyl transferase 1

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The cytoskeleton

I. The cytoskeleton

The cytoskeleton is a network of filaments and fibres present in all eukaryotic cells. It is composed of three main elements: microfilament of actin, microtubules (MTs) and intermediate filaments (IFs). To maintain cellular homeostasis, every component of the cytoskeleton plays a different and important role (Fletcher & Mullins, 2010). The filaments give a track for protein and organelle transport leading to the organisation of the cellular space. The cytoskeleton connects the cell to the extracellular matrix and exerts forces to regulate the shape of the cell. The cytoskeleton provides mechanical support and is actively involved in essential pathways controlling division and migration.

I-1. Actin

I-1-1. Form and assembly

Actin can be considered an active biopolymer due to actin binding protein (ABP) cross binding the networks in addition to molecular motors found along the filament. Actin growth is a polar process, with monomeric G-actin molecules being added at one end of the filament. Indeed, actin is part of a globular protein family that forms microfilament and is present in all eukaryote cells. Actin has three different isoforms: α -actin in skeletal, cardiac and smooth muscle cells and β - γ - isoforms in non-muscle and muscle cells. Actin is present in two different states in the cell: as an available pool of monomers the G-actin and as a filament F-actin. G-actin assembles into a 5-9nm large F-actin filament in an ATP dependent manner at the positively charged barbed end of the filament (Roberto Dominguez and Kenneth C. Holmes, 2011) (Figure 1).

I-1-2. Different structures of actin

Actin can form different structures within the cell. It assembles into a higher-order structure as bundles or 3D networks having similar physical properties as semisolid gels. Actin bundles accumulate at the plasma membrane with actin-binding protein in a 3D network called the cell cortex, or cortical actin. This structure gives the cell its shape and is important to several processes at the cell surface (Cooper, 2000b). The polymerisation of actin against the plasma membrane produces forces used for membrane protrusions or invagination. Actin filaments composed the major contractile structure of the eukaryote cells. To produce forces against the extracellular substrate, actin together with myosin II filaments assemble into stress fibres (Tojkander et al., 2012a). Stress fibres are contractile bundles of 10-30 actin filaments held in place by α -actinin. Depending on their morphology and localisation, stress fibres are differently categorised: there are dorsal and ventral fibres, transverse arcs and perinuclear actin caps (Tojkander et al., 2012b). Dorsal fibres do not contain myosin II and therefore do not contract but are associated with Focal adhesions (FAs). Stress fibres were described in most cultured

cells, especially endothelial. They play an essential role in adhesion mechanism anchoring FA structure. The mechanical properties of the actomyosin network are described further in Chapter II. The structure of actin filaments and bundles is tightly dependent on its post-translational modifications (PTM).

I-1-3. Post-translational modifications

Actin structure, folding and interaction with other actin-binding proteins, and therefore actin dynamic, are all affected by PTMs (Varland et al., 2019). The first described PTM in actin is the N-terminal acetylation (Gaetjens & Bárány, 1966) and there is today at least 140 types of PTMs discovered on actin, with the main ones being methylation, acetylation, arginylation, SUMOylation, ubiquitination, ADP-ribosylation, methionine oxidation, cysteine oxidation, phosphorylation, and tyrosine nitration. Acetylation of actin allows for stronger actomyosin interactions. It might also determine subsequent ubiquitination and consequently its metabolic fate. Acetylation is extremely important for the maturation and maintenance of the actin structure. Acetylation is mediated by different acetyl-transferase and deacetylase enzymes. The Histone Deacetylase 6 (HDAC6) is thought to interact with actin to take away the acetyl group that would lead to active actin rearrangement *in vivo* (Terman & Kashina, 2013).

Actin is also methylated at His-73. This particular modification affects the flexibility and stability of actin. The methylated group on His-73 is thought to slow down the release of inorganic phosphate after the hydrolysis of ATP, stabilizing F-actin (Terman & Kashina, 2013). Actin can also be ubiquitinated, by several enzymes such as MuRF-1, UbcH5, and Trim32, that tag actin for degradation, inducing a decrease in actin levels (Kudryashova et al., 2005). Hence, PTMs are very important in the organisation of the actin network for different cellular processes.

I-2. Microtubule

I-2-1. Form and assembly

MTs result from the assembly of polar and linear protofilaments composed of the two subunits of tubulin α and β . They form hollow tubes of 25nm diameter. They have two structural conformations closely linked to their function. The linear proto filament provides a substrate for molecular motors and the bent filament gives the MT a more rigid conformation, affecting the growth of the filament (Sept et al., 2003). Regardless of their rigidity, MTs are by themselves very dynamic. They alternate periods of shrinkage and growth called dynamic instability (Figure 1) (Mitchison & Kirschner, 1984). In growth, the addition of GTP-tubulin dimers leads to the formation of a stabilising cap at the plus-end of MTs. The loss of this cap induces MTs to shrink because of the very unstable shaft under the cap when GTP-tubulin is hydrolysed into GDP-tubulin (Akhmanova & Steinmetz, 2015). This hydrolysis releases energy, which speculatively goes to deform the tubulin filament giving it a curvature making the lattice more stable. When

the energy stored is released and the cap unstable, the shrinking phase is starting (Burbank & Mitchison, 2006).

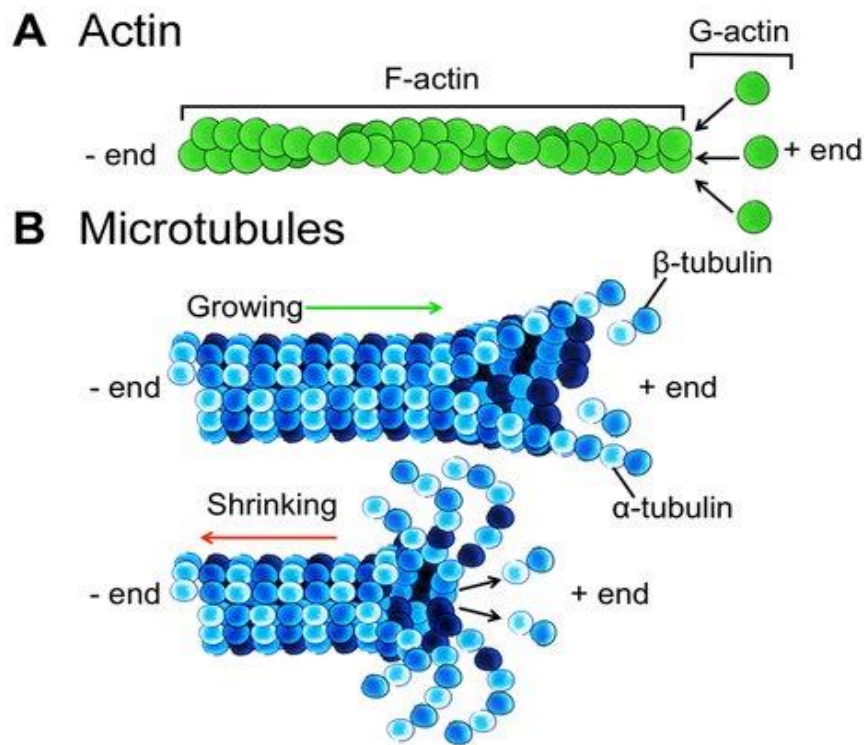


Figure 1 Form and assembly of actin and microtubules. Actin and microtubule filaments grow in a polar manner by adding monomers to the plus ends of the filament. Additionally, microtubules go through dynamic growing and shrinking phases. (Adapted from Motony, 2014)

MTs mainly nucleate from MT organising centre (MTOC), mostly the centrosome. Being polar filaments, cargoes are transported alongside them thanks to molecular motors such as kinesins, more commonly transporting cargoes from the minus towards the plus-end of the MT and dyneins moving in the opposite direction (Nogales, 2000). Positioning of the MTOC allows reorganisation of the network during the different cellular events, such as cell division and cell migration.

MTs are important for several cellular processes such as migration (Etienne-Manneville, 2013; Garcin & Straube, 2019; Seetharaman et al., 2020; Watanabe et al., 2005) but have been extensively studied in cell division. During cell division, the mitotic spindle formed by a dense array of MTs mediates the separation of chromosomes into the two daughter cells. The MTs within the mitotic spindle exert forces to segregate the chromosomes and move them towards the opposite poles of the cell (Forth & Kapoor, 2017a).

I-2-2. Post-translational modifications

MTs undergo a surprisingly high number of PTMs, affecting their function and organisation within the cell as seen in figure 2. PTM can affect the MTs (in blue on the table) or the tubulin subunit (in red). The PTM discovery journey began almost 50 years ago with the discovery of RNA-independent enzymatic incorporation of tyrosin (Barra et al., 1973).

One of the most important PTM is acetylation occurring at a K40 residue of alpha-tubulin inside the lumen of the MT. Acetylation is mediated by several enzymes: the addition of the acetyl group is performed by alpha-tubulin Acetyl Transferase 1 (α -TAT1), and the removal is assured by NAD-dependent deacetylase sirtuin type 2 (SIRT2) and HDAC6, which, unlike its name indicates, has alpha-tubulin as the primary substrate. Acetylation of MT is important in different cellular processes, especially cell migration by increasing within the vicinity of FAs in astrocytes, promoting an increase of FA turnover and in turn astrocyte migration (Bance et al., 2019). For more details, see III-4-2.

Modification	α/β	Site	Enzyme
Acetylation	α	Lys40	TAT(MEC17)
Deacetylation	α		HDAC6, SIRT2, HDAC5
Detyrosination	α	C-terminal Tyr	Unknown
Tyrosination	α	Add to C-terminal	TTL
$\Delta 2$ deglutamylation	α	Removal of C-terminal Glu of detyronisated MTs	CCP1, CCP2, CCP3, CCP4, CCP5 and CCP6
Glutamylation	α/β	Various C-terminal Glu	TLL4, 5 and 7
Deglutamylation of branch point Glu	α/β		CCP5 and CCP1, CCP4, CCP6
Polyglutamylation	α/β	Add γ -linked Glu to C-terminal	TLL1, 6, 11 and 13
Deglutamylation to shorten polyGlu side chain	α/β		CCP1, CCP4, CCP6
Glycylation	α/β	Add γ -linked Gly to C-terminal Glu	TLL3 and 8
Polyglycylation		Addition to γ -linked Gly	TLL10
Deglycylation		C-terminal	Unknown
Polyamination	α/β	β tubulins Q15, others	Transglutaminase
Glycosylation	α/β	various	unknown
Glycation	α/β	various	Nonenzymatic
Palmitoylation	α	Cys376	unknown
Phosphorylation	α/β	β tubulin Ser172, others, others	various
Sumoylation	α	multiple	unknown
Ubiquitylation	α	multiple	Parkin

Figure 2 PTM of MTs. In blue are the PTM of MT, in red the one done on the subunits and in black are either done on both or unknown (adapted from Song & Brady, 2014).

I-3. Intermediate filaments:

Intermediate filaments characterisation began with their discovery in 1928 as described in the third edition of the textbook “The cell in development and heredity” (Wilson, 1928) and followed by the discovery of the actual structure of the keratin in 1952 and since then the research and discovery of IF proteins have only increased.

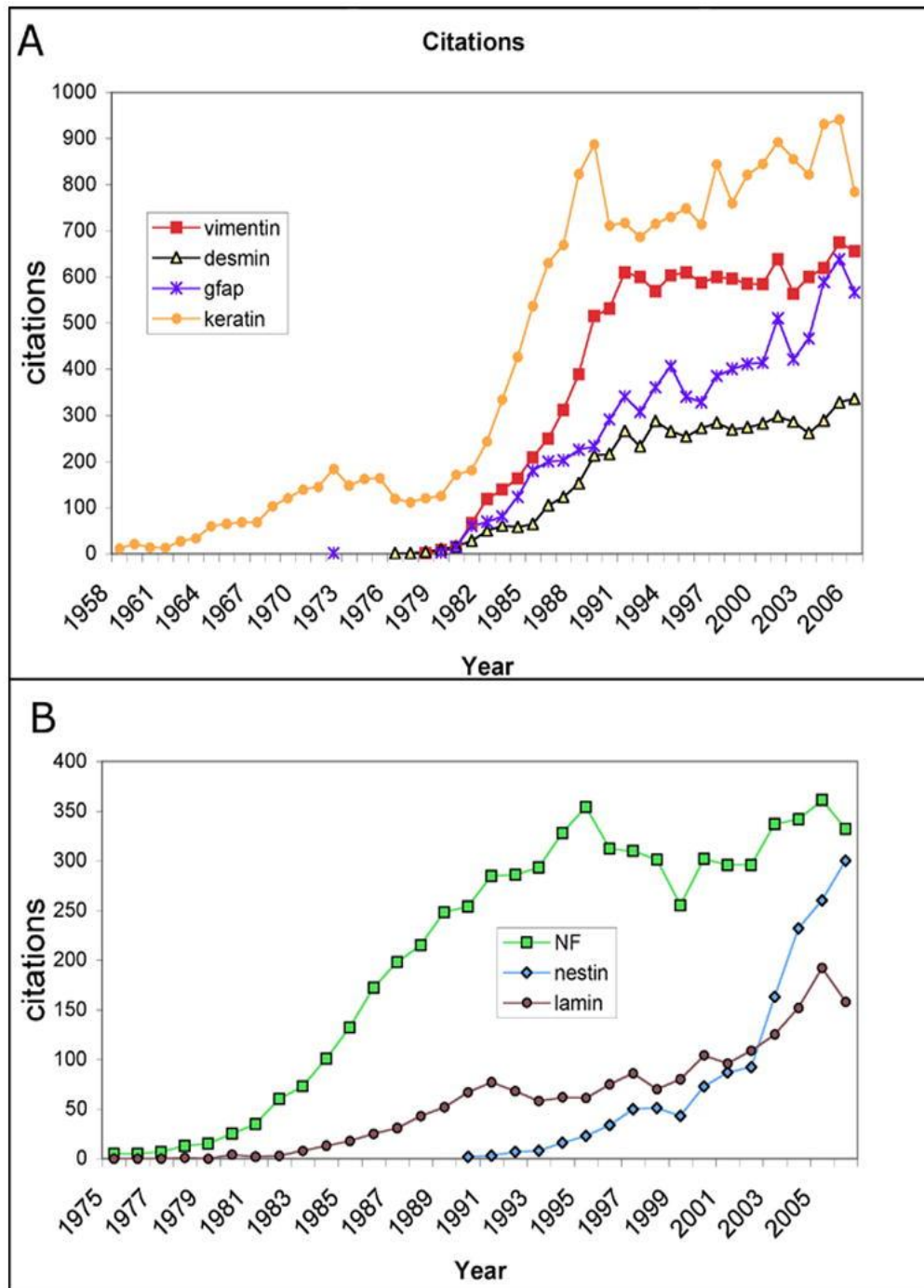


Figure 3 Number of publications on IFs per year from their discovery to 2006 (Oshima, 2007). Research started to take off but are plateauing after main discovery are made.

The discovery of keratin was followed that of GFAP and research took off after the characterisation of vimentin in 1978 (Eng et al., 1971; Hynes & Destree, 1978). The main characterisation and advancement in the IFs field started in the '80s (Figure 3). Nonetheless, there is still a lot to understand in their physical properties and biochemical interactions in cells. The following part will describe the general properties of IFs.

I-3-1. Structure of IF proteins

Cytosolic IFs (cIFs) derive from a common ancestor with the more ubiquitously expressed nuclear filament Lamin, traced back to a species outside of the metazoan lineage: the slime mould *Dictyostelium* (Peter & Stick, 2015a). The slime mould presents a protein NE81 with several of the key features that make up lamins at the exception of a highly conserved IF consensus at the end of the rod domain. This allows pushing the origin of the lamins to the uniconts (Peter & Stick, 2015b).

IF proteins have a uniform, global structure based on a common central domain. The first X-ray crystal structure of an IF protein — keratin — was reported in 1932 by William Astbury and led, through Linus Pauling's α -helix model, to the prediction of the coiled-coil structure of keratin by Francis Crick in 1952 (Crick, 1952). This common α -helical rod domain of approximately 310 amino acids is present in all IF proteins. It is flanked by head and tail domains of diverse size and structures that characterise each IF protein and allow them to interact with specific intracellular partners (Dutour-Provenzano & Etienne-Manneville, 2021).

I-3-2. Assembly into filament

IFs are commonly depicted as a molecular scaffold organising the cellular space. To build this scaffold, the cell is drawing from a soluble tetramer protein pool. Since IFs are highly stable, in

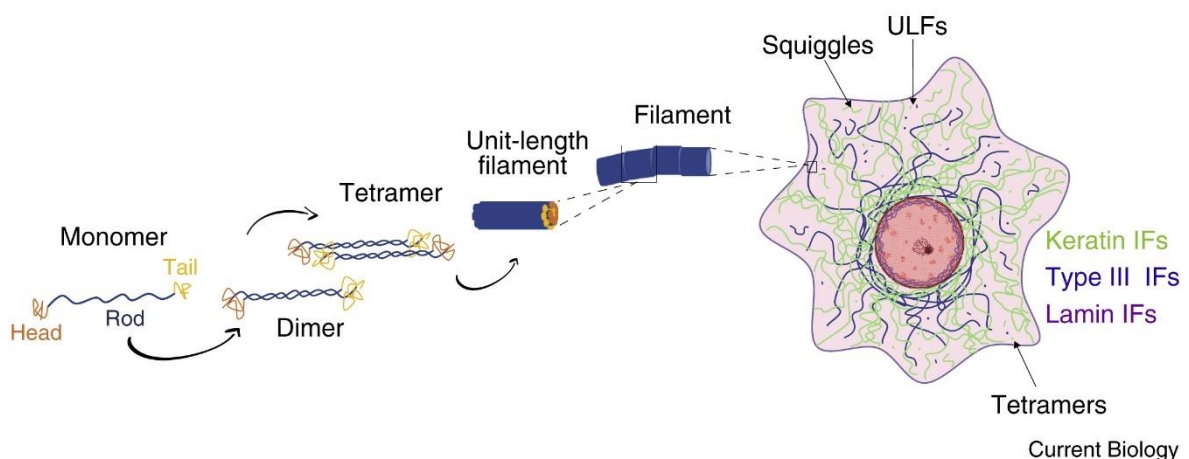


Figure 4 IFs assembly. Monomers of IFs assemble into a dimer through an interaction between their rod domains. Dimers assemble in an antiparallel manner to form soluble tetramers, which in turn assemble into unit length filaments (ULFs) that, via end-to-end binding, elongate into filaments. Soluble tetramers, ULFs, and squiggles, which correspond to short IFs and long IFs, can all be found in cells.

a wild type situation, the pool is barely detectable and represents only 1-5% of the total protein (Lodish et al., 2000; Robert et al., 2016). The pool of tetramers formed by the assembly of dimers into the three-partite structure is rapidly drained to produce octamers, an obligatory step before the formation of Unit Length Filaments (ULF) leading to a complete filament (Mücke et al., 2018). When the assembly is disrupted and stopped at the ULF stage, the tetramer pool increases by 50%. Thus, the subunit exchange between the ULFs is a dynamic process (Robert et al., 2015). The assembly is depicted in figure 4.

I-3-3. Classification of IF proteins

I-3-3-1. Differences between nuclear and cytoplasmic IFs

There are two distinct types of IFs: the cytosolic and the nuclear (Figure 5) (Kornreich et al., 2015). The nuclear meshwork of intermediate filaments lay under the nuclear lamina and contribute to the nucleus shape. The cytosolic IFs extend between the periphery of the cell and the nucleus. The structure of Lamin is distinct from cytosolic IFs as a result of an NLS and a CAAX box motif that allows the protein to target the inner nuclear membrane (Figure 5). cIFs are derived from the loss of these two structural features, which banishes them from the nucleus. CIFs have a standard diameter of 10 nm, where nuclear IFs form by concomitant lateral and longitudinal, head to tail association of the dimers, generating filaments with variable diameter and length. This account for the irregular filamentous meshwork formed by lamins in comparison to the regular and long filaments observed in the cytoplasm. Each lamin component (A/C, B) forms its own meshwork (de Leeuw et al., 2018). The differences between IF proteins leading to their differential categorisation occur within the head and tail domains (Cooper, 2000a; Lodish et al., 2000) (Herrmann et al., 2007).

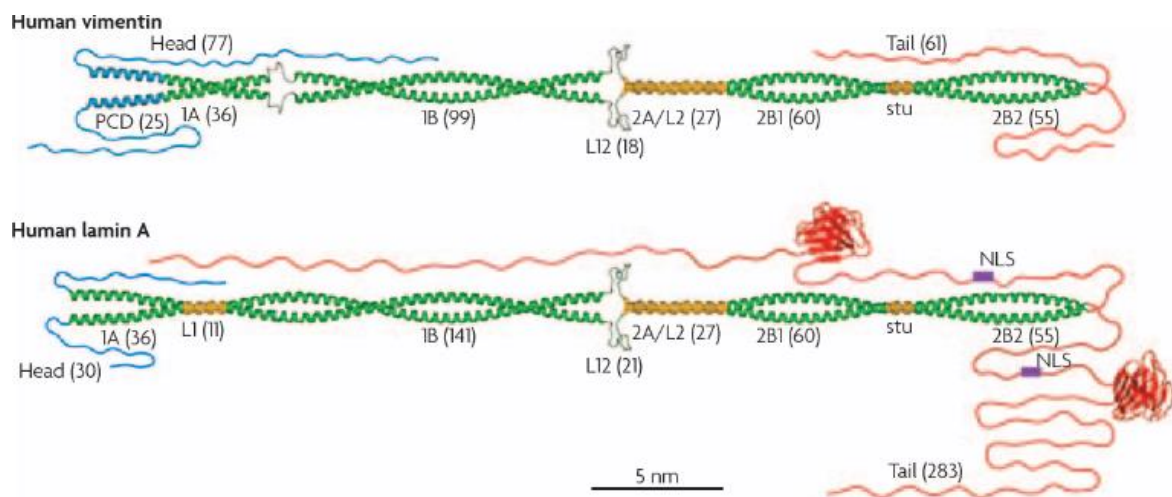


Figure 5 Different structure between cytoplasmic IF vimentin and nuclear IF lamin A. Nuclear IFs distinguish themselves thanks to an NLS sequence and a CAAX box motif that allow their entry in the nucleus and localise them at the envelop. Nearly parallel helical bundle are represented in yellow, left handed coiled-coil region in green and non-helical linker in grey (Herrmann et al, 2007).

I-3-3-2. Different categories of IF proteins

The different IF proteins are classified into six categories differentially expressed within the cell types. Type I and II comprise the keratins, the most abundant form of IFs, which are highly expressed in epithelial cells. The Type III proteins are expressed in various cell types (fibroblast, endothelial, glial and muscle cells) and are comprised of Vimentin, Desmin, Glial Fibrillary Acidic Protein (GFAP), as well as peripherin (Etienne-Manneville, 2018). This category of IFs is the only category that can self-assemble and form homopolymers as well as heteropolymers with other IF proteins from type III and type IV. The mechanism by which some filaments can form homopolymers and other such as Keratin are obligate heteropolymers remains to be elucidated. Type IV, including Nestin, Synemin and neurofilaments are mainly expressed in neurons and glial cells. Type VI includes lens-specific IFs. The nuclear lamins are represented in group V (Figure 6).

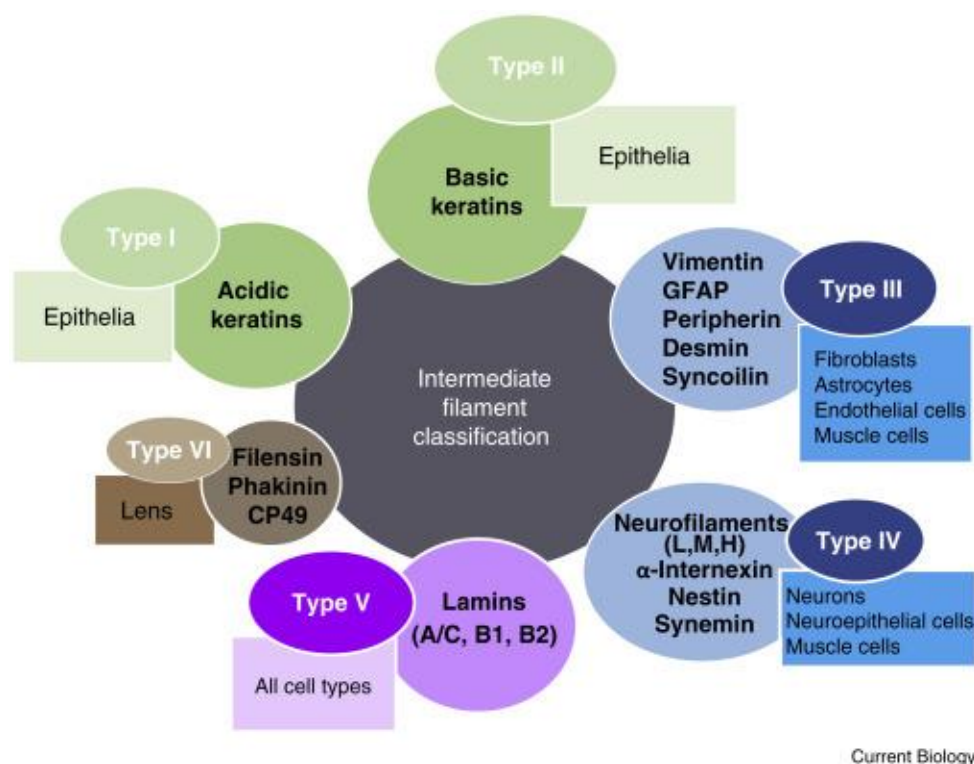


Figure 6 IFs classification. Type I and II proteins correspond respectively to acidic and basic keratins, the most abundant IF proteins, which associate in obligate heteropolymers that are highly expressed in epithelial cells. Type III proteins are expressed in a variety of cell types, including fibroblasts, and endothelial, glial and muscle cells, and comprise vimentin, desmin, glial fibrillary acidic protein (GFAP), as well as peripherin. Type IV proteins, including nestin, synemin, α -internexin, syncoilin and neurofilament proteins, are mainly expressed in progenitor or differentiating cells and also in neuronal and glial cells. Type V proteins correspond to the ubiquitously expressed lamins, which contain a nuclear localization sequence and a CAAX box motif that targets the protein to the inner nuclear membrane. Finally, type VI represents a group of very divergent proteins, including the lens-specific IF proteins (Bfsp1/filensin, Bfsp2/phakinin and CP49) (Dutour Provenzano & Etienne-Manneville, 2021).

I-3-3-3. Alteration and mutations

IF-related diseases affect particular tissues or organs that mostly correspond to the sites of expression of the particular IF proteins. They are well known to disturb the assembly of IFs. More than 90 pathologies, including the so-called laminopathies, keratinopathies and desminopathies, have been associated with mutations in IF proteins (Omary et al., 2004). Mutation of desmin, which is specifically expressed in muscle cells, impacts muscle function causing skeletal myopathies or cardiomyopathies (Goldfarb et al., 2004). In contrast, mutations in keratins, which form a large family of IF proteins differentially expressed as sets of one, two, or three pairs in diverse epithelia, give rise to several skin, hair, lens and liver disorders (Dutour-Provenzano & Etienne-Manneville, 2021).

IF-related diseases are caused by mutations that generally lead to the reorganization but not the loss of the IF network. For instance, heterozygous mutations in the type III GFAP gene that cause Alexander disease engender the accumulation of IF proteins in proteinaceous aggregates called Rosenthal fibres in astrocytes (Brenner et al., 2001; Sosunov et al., 2017). More specific mouse models expressing mutated IF proteins have confirmed that the altered organization of the IF network plays a key role in these pathologies. Finally, IF-related diseases and *in vivo* models point to an essential role for IFs in tissue mechanical resistance and tissue integrity (Dutour-Provenzano & Etienne-Manneville, 2021; van Bodegraven & Etienne-Manneville, 2021).

I-3-4. Post-translational modifications

I-3-4-1. Phosphorylation

Vimentin post-translational modifications

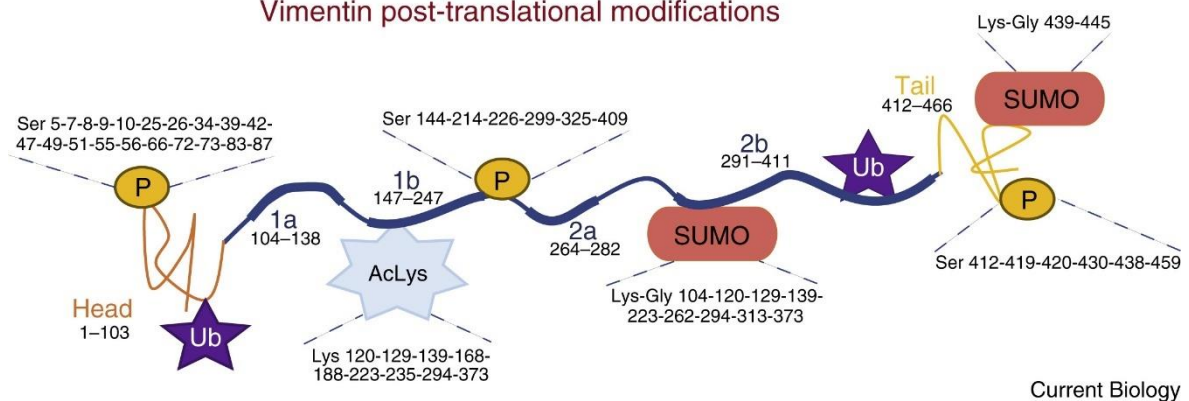


Figure 7 IF Post-translational modifications. The major post-translational modifications, including phosphorylation, sumoylation, acetylation and ubiquitination, that are known to affect vimentin are shown. As for all IF proteins, post-translational modification occur throughout the vimentin protein and affect the dynamics, mechanics and biochemical properties of the resulting filaments.

IFs undergo different post-translational modifications with phosphorylation being the main modification (Figure 7). It occurs mainly during mitosis and leads to a complete IF network

reorganisation. The first enzyme found to phosphorylate IF Vimentin is the Protein Kinase A (PKA). PKA mediated phosphorylation of Vimentin leads to complete disassembly of the network. The majority of phosphorylation sites are found in the head domain or N-terminal end. The head of the monomer is usually positively charged due to basic residue present, being necessary to the IF assembly (Goto & Inagaki, n.d.). Phosphorylation at this site changes the electric charge thanks to the phosphate group addition. Phosphorylation of IFs is site-specific and is dependent on the type of filament (Figure 8, Table 1) (Tokui & Takahashi, n.d.). Phosphorylation of IFs leads to the formation of thick bundles and the collapse into spheroid aggregate bodies and fragmentation of the filament. A defect in phosphorylation of IFs is featured in several diseases such as Alzheimer, Parkinson and motor neuron disease where neurofilaments are abnormally phosphorylated and found in aggregates (Sihag et al., 2008). In general, phosphorylation of IFs helps the reorganisation of filament network necessary for important physiological pathways and thus facilitating subunit exchange, typically at a Ser/Thr residue. Several IFs binding proteins exclusively bind phosphorylated groups at Ser/Thr residues. This is the case of 14-3-3 proteins, and the interaction leads to cell growth and tumorigenesis. The binding of this protein to keratin 18 leads to activation of mTORC pathway and subsequently, cell growth (Ku et al., 2002) and binding of 14-3-3 protein to vimentin inhibits

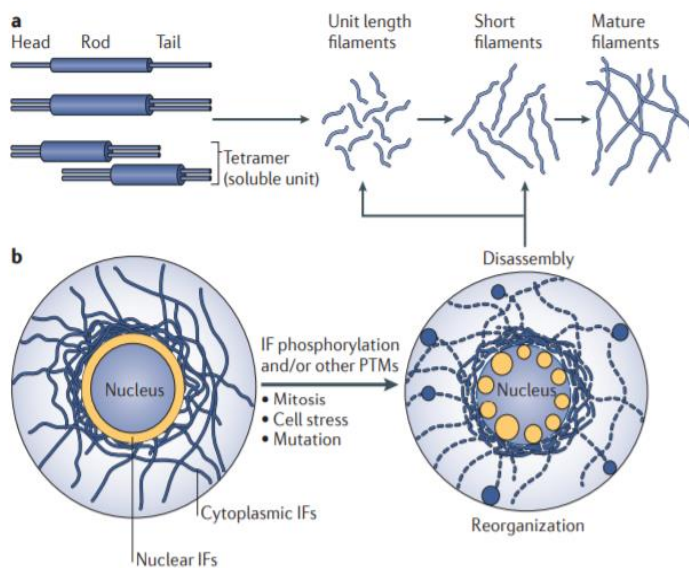


Table 1. In vitro disassembly of IF proteins by phosphorylation

IF	Kinase	Disassembly
Vimentin	A kinase	+
	C kinase	+
	CaMKII	+
	cdc2 kinase	+
	G kinase	-
	Casein kinase I	-
	Casein kinase II	-
GFAP	A kinase	+
	C kinase	+
	CaMKII	+
	cdc2 kinase	-
Desmin	A kinase	+
	C kinase	+
	cdc2 kinase	+
Keratin 8/keratin 18 complex	A kinase	+
	C kinase	+
	CaMKII	+
α -Internexin	A kinase	+
NF-L	A kinase	+
	C kinase	+
	cdc2 kinase	-
Lamin B	cdc2 kinase	-
	C kinase	+/-
	A kinase	-
	MAP kinase	+

Figure 8 PTMs effect on reorganisation. IFs are dynamic structures that need to reorganise under certain physiological conditions such as migration, division and in response to mutation. Phosphorylation might be the most important PTM for the organisation of the network. Certain phosphorylation leads to the disassembly of the filament. The phosphorylation is mediated through different kinases depending on the kind of IF, as seen in table 1 (Snider and Omary, 2014).

autophagy and promote AKT-mediated tumorigenesis pathway. This activation can therefore promote migratory behaviour in the cell (Snider & Omary, 2014) (More details about 14-3-3 and IFs in II-3-2).

I-3-4-2. Farnesylation, glycosylation, transglutamination

Other important post-translational modifications are Sumoylation, Lys acetylation, glycosylation and Farnesylation. Sumoylation, like phosphorylation, promotes the organisation and solubility of cytoplasmic IFs. Hypersumoylation is featured in apoptosis, oxidative stress and chronic liver disease. At the opposite of phosphorylation and sumoylation, acetylation at Lys residue in keratin 8 helps its stabilisation by reducing its solubility and promoting a denser network around the nucleus. SIRT2 is introduced as a broad regulator of IFs acetylation (Snider & Omary, 2014). An interesting modification is the oxidation of cystein in vimentin. The oxidation of the single cystein residue (C328) allows for vimentin reorganisation and growth of the network. C328 associate with zinc and allows the filament to polymerise. A zinc deficiency leads to higher solubility of the vimentin network (Pérez-Sala et al., 2015).

Note:

PTMs of IFs are key to determine the organisation of the network, the most important being phosphorylation, leading to the solubility of the network, important in several cellular processes like cell division.

I-4. IFs in Astrocytes

I-4-1. Description of the astrocyte model

The mammalian brain is composed of two main types of cells: the neurones and the glia. These two types are in constant interaction but the glial population is more abundant. 20 to 40% of the cells composing the glia are astrocytes. Astrocytes take their name after their star-like shape (*Astron* meaning star and *kytos*, a hollow vessel) (Parpura & Verkhratsky, 2012). The first observation of glia was made in 1858 by Rudolf Virshow, but he described it as a substance shaping nervous parts rather than proper cells. At the end of the 19th century, pioneer Camillo Golgi, first characterised glia as proper cells, through silver staining (Figure 9) (*Opera Omnia / Camillo Golgi*, n.d.; Potokar et al., 2020).

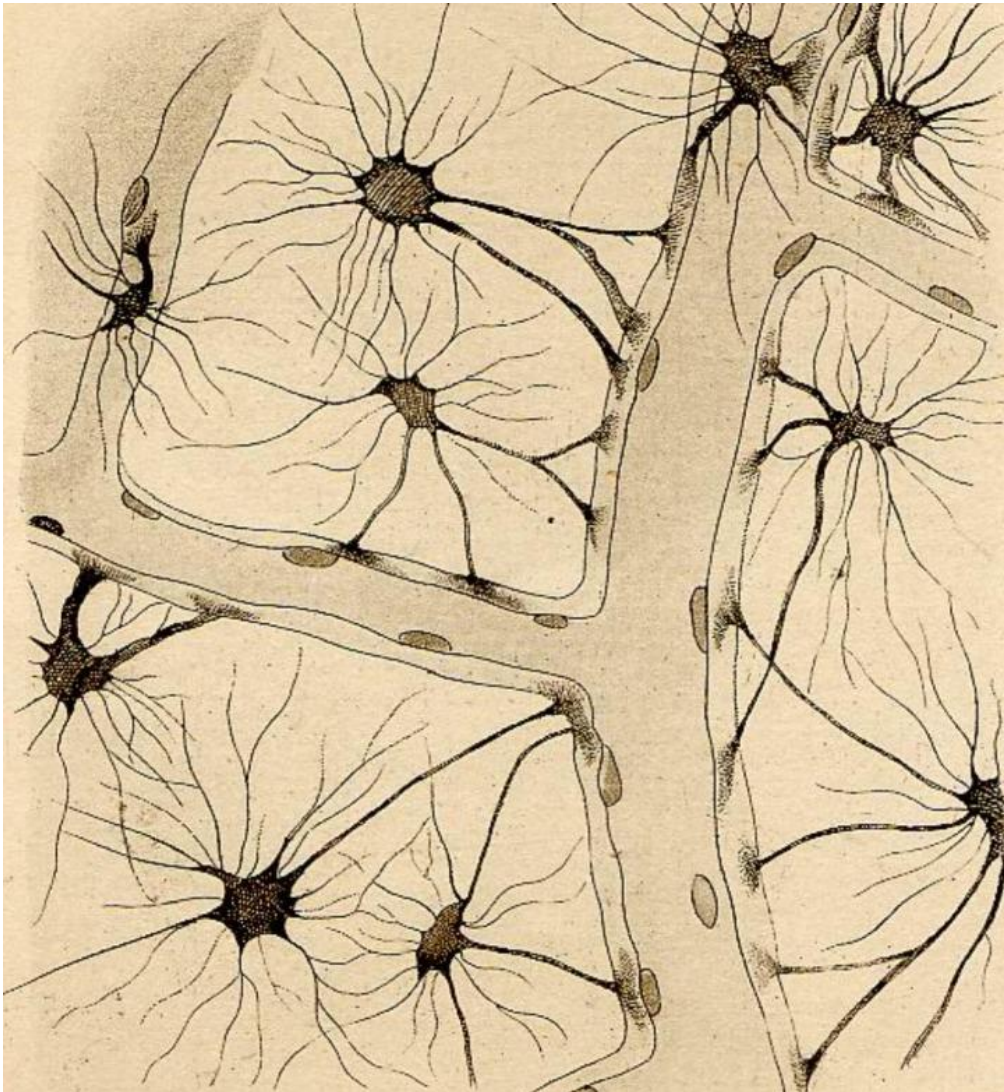


Figure 9 Neuroglial cells drawn by Camillo Golgi, reproduced from cells stained using silver chromate technique (Golgi, C. 1903).

The name astrocyte, however, was first given in 1891 by Mihály Lenhossék, a Hungarian anatomist and histologist. *In vivo*, astrocytes form long protrusions, wrapping themselves around

neurones. Astrocytes possess variable morphology and function in the brain (Parpura & Verkhratsky, 2012).

Their main function is to create a supportive network for the neurones, bringing them energy delivered through the astrocyte-neuron lactate shuttle (Bass, 1971). Astrocytes are extremely important to maintain extracellular homeostasis in the brain (Siracusa et al., 2019). They are like the orchestra director of the brain by ensuring the good conduction of the signal transmission at synapses as well as their formation.

Elimination of neuronal metabolites. They act as the housekeeper of the brain: neurons depend on astrocytes to eliminate and neutralise excessive Reactive Oxygen Species ROS in the brain. To do so, they possess antioxidant systems such as the GSSG-GSH system, peroxidase enzyme, dismutase and catalase to maintain neuronal health (Kimelberg & Nedergaard, 2010).

Control of cerebral flow. Astrocytes, along with endothelial cells and pericytes, are the main type of cells composing the blood-brain barrier. Their processes englobe every blood vessel and therefore participate in the regulation of traffic of molecules through the blood vessel walls. Established during development, this cellular association persists at maturation and control the diameters of the vessel by constriction through the release of vasodilations or constriction molecules (Kimelberg & Nedergaard, 2010; Sofroniew & Vinters, 2010).

Synaptic transmission. Astrocytes processes wrap themselves up around the pre and post-synaptic neuronal structure as they sense the synaptic activity and respond to it via intracellular Ca^{2+} transient (Figure 10) (Eroglu & Barres, 2010). A single human astrocyte comes in contact with up to 2,000,000 synapses through their fine presynaptic processes (PAP) (Bushong et al., 2002; Oberheim et al., 2009). Astrocyte presence at synapses is crucial as they allow for the regulation of transmitter release such as glutamate, purines, GABA and D-serine (Sofroniew & Vinters, 2010). To associate to the synapse, they possess adhesive molecules: the best candidate is the EphA4 receptor tyrosine kinase that localises at the dendritic spine of hippocampal pyramidal neurons. Its ligand Ephrin-A3 appears on the PAP membrane. Impairment of this binding leads to unstable spines with disrupted shapes (Nishida & Okabe, 2007). Most importantly, N-cadherin mediated adhesion allows for the stability of the neuronal connections. N-cadherins are Ca^{2+} dependent cell-cell adhesion molecules (Hirano & Takeichi, 2012). The actin cytoskeleton, Ca^{2+} sensitive, is mainly present in the PAP and aggregation of actin-binding protein alpha-actinin is observed at the tip of the filopodia (Safavi-Abbasi et al., 2001). N-cadherins are linking the actin network for tension allowing correct adhesion. This might be allowed by the capacity of astrocyte to elevate Ca^{2+} levels at synapses, in turn, used to trigger

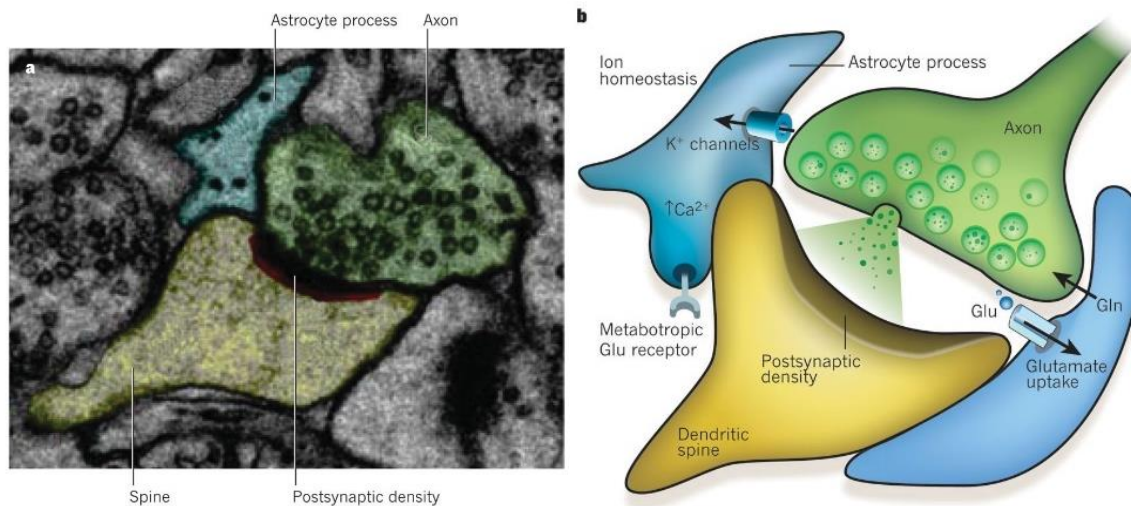


Figure 10 Astrocytes present at the synapse. Astrocytes processes are present at synapses and play a structural and functional role. In the electron micrograph is showing the tripartite component with the astrocytic process in blue and presynaptic axon in green and post synaptic dendritic spine in yellow. b is a schematic representation of the synapse. Astrocyte processes have glutamate transporter that intake glutamate (Glu) released in the synapse and transport it back to the neuron transformed in glutamine (Gln). The astrocytes are regulating the ion concentration and the pH to allow good transmission of the signal (Eroglu & Barres, 2010).

the release of vesicular transport of glutamate (Guerra-Gomes et al., 2018). This allows the good functioning of the synapse. Once again, the cytoskeleton might play a key role in this astrocytic function.

The role of the cytoskeleton is crucial in all the functions of astrocytes. To achieve those functions, they need a specific set of expressed cytoskeletal genes. This is an extremely important step helping with the changes in metabolism and the adaptation to different environments. We want to give the focus here on intermediate filaments. Indeed, astrocytes express a specific combination of IFs.

I-4-2. Different IFs present in Astrocytes:

Astrocytes express four cIF proteins. GFAP and vimentin are the most abundant, and Nestin and Synemin are expressed in the form of heteropolymers associated with GFAP and/or vimentin.

I-4-2-1. GFAP

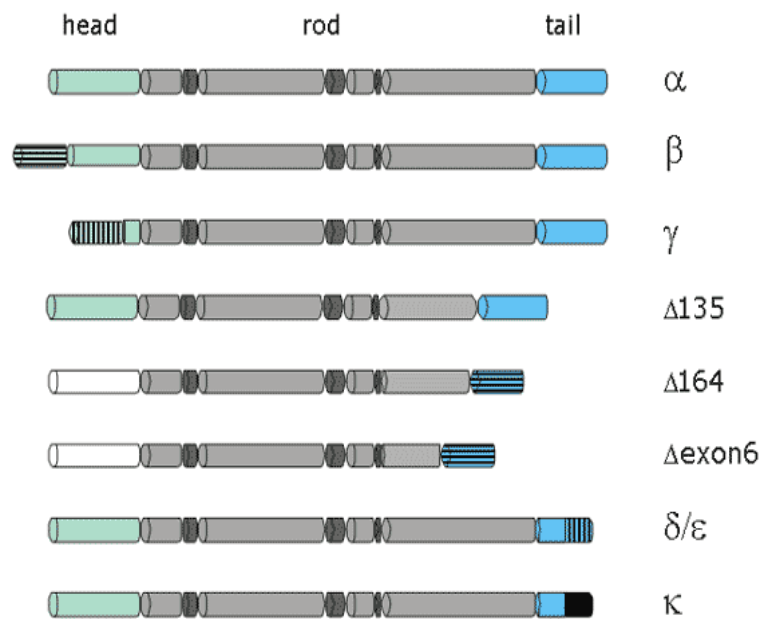
GFAP is a type III IF. Several cells can express GFAP but at almost undetectable amounts, whereas it is highly expressed in mature astrocytes. This makes GFAP a specific marker for astrocytes. It was first discovered in 1971 by Eng *et al* from three different samples: multiple sclerosis plaques, post leukotomy scars and the periventricular corneal layer tissues. The filament then observed, showed different physical characteristics from other fibrillary proteins expressed in glial cells, setting GFAP apart from, for example, tubulin (Eng et al., 1971). The discovery of

GFAP was key to study the function of astrocytes, in terms of morphology, motility and stability of astrocytic processes, within the Central Nervous System (CNS) (Bignami et al., 1972; Tykhomyrov et al., 2016).

GFAP, after keratins, is the IF having the most isoforms. At least 8 isoforms are arising from splice variation, expressed in specific types of astrocytes (Figure 11). The most abundant form is GFAP α and was the first one identified. Most works related to GFAP are describing this isoform. The human CNS is expressing all the isoforms except GFAP β only found in rats (Figure 11) (Middeldorp & Hol, 2011; Yang & Wang, 2015).

Animal models. Several GFAP negative animal models were generated to better study the role of GFAP in the CNS. No embryonic lethality is observed in these GFAP^{-/-} mice. The mice seemed at first to develop healthy, without behavioural issues and no motor incoordination. The GFAP^{-/-} mice are fertile (Gomi et al., 1995). They are, however, more susceptible to the infection of scrapie prions. Astrocytes of the mice model generated by Pekny *et al* (1995) presented a complete lack of intermediate filaments, showing no upregulation of other IFs upon ablation of GFAP. They also presented reactive gliosis, showing that, although GFAP upregulation is a hallmark in this process, it might not be necessarily required (Pekny et al., 1995). After further studies from the same group, they found that GFAP devoid cells can express vimentin and nestin, although a reduction of the level expressed is noted. In whole-brain culture, GFAP^{-/-} exhibit a higher level of proliferation (Pekny et al., 1998).

A third mouse model has seen the day the following year, presenting, as for the other models, normal development and fertility. The researchers focused on the interaction between astrocytic processes and synapses in the hippocampus region of the brain. They assessed the potentiation at pre and postsynaptic region, and saw an enhanced long term potentiation in the GFAP negative mice compared to the control, suggesting that GFAP in the astrocytes process is important for the interaction with the synapse, modulating synapse efficiency in the CNS (McCall et al., 1996).



GFAP isoforms human accession no.	Localization	a.a.	Features
Gfap-α (Isoform 1) NP_002046.1	-Main isoform in astrocytes in CNS, -Also in schwann cells PNS	432	Predominant isoform in brain
Gfap-β	-High levels in PNS- schwann cells	>432	-Included sequence before exon-1; -Exact start site unknown
Gfap-γ	-Corpus callosum -mRNA found in brain, bone marrow, spleen	<432	-Included sequence before exon-1, omission of exon-1; included intron before exon-2 -Exact start site unknown
Gfap-δ /GFAP-ϵ (Isoform 2) NP_001124491.1	-Preferentially expressed by neurogenic astrocytes (SVZ) -Potential binding to presenilins	431	-Included intron before exon-8, alternate C-terminal
Gfap-κ (kappa) (Isoform 3) NP_001229305.1	-Transcripts can be found in mouse cortex, striatum and cerebellum	438	-Included intron before exon-8; alternate C-terminal
Gfap-ζ (zeta)	-Newly identified isoform	>432	Included intron before exon-9
GFAP +1	GFAPΔEx6	347	Skipped exon-6; lacking coil 2B -Exact start site unknown
	GFAPΔ164	366	-Shortened exon-6, 7; lacking coil 2B -Exact start site unknown
	GFAPΔ135	374	-Shortened exon-6; lacking coil 2B
	GFAPΔEx7	418	-Skipped exon-7 -Exact start site unknown

Figure 11 Different isoforms of GFAP. GFAP is spliced alternatively and give rise to 8 isoforms. The most abundant and well-studied is the α isoform. As seen in table1, the isoforms are present in different cell type but only in the brain, with astrocytes being the predominant cell type. (Yang & Wang, 2015; <http://www.elhybollab.eu/research/gfap>).

GFAP functions. In a study by de Pablo et al (2013), they found that mice astrocytes deprived of Vimentin and GFAP are more sensitive to Oxygen Glucose Deprivation than the wild type (de Pablo et al., 2013). After coculture of neurons and astrocyte WT or VIM^{-/-}GFAP^{-/-}, the cells were treated with OGD condition for 18hours. An increased neuronal death was observed in VIM^{-/-}GFAP^{-/-} compared to the control. This result attributes a neuroprotective role to vimentin and GFAP. It is also noted that after stress induction by Reactive Oxygen Species (ROS), their elimination is impaired in VIM^{-/-}GFAP^{-/-}. Loss of GFAP is also associated with increased susceptibility to percussive head injury: in a study using a weight drop device, GFAP null mice died upon percussion within a few minutes, whereas the wild type could survive (Nawashiro et al., 1998). This gives GFAP protective properties upon mechanical stress injunction.

GFAP expression is increased in reactive gliosis. To better understand the effect of this increased expression, transgenic mice overexpressing human GFAP genes were created. GFAP overexpression in astrocytes leads to their hypertrophy, overexpression of the small heat-shock proteins like α B-crystallin and HSP25 (N.-H. Lin et al., 2021) and most remarkably, the same Rosenthal fibres inclusion bodies observed in Alexander disease. This mouse line provides a good model to study the GFAP-related disease, explained in the following part. The generation of KO mice for both GFAP and vimentin, exhibit reduced astroglial reactivity (Figure 12). As GFAP null cell alone have normal reactive gliosis, it suggests that both IFs are necessary for the induction of reactive gliosis. Glial scar stands in the way of the neuron being able to repair its axon after injury, forming biochemical and physical barriers (Horvat, 1992). Therefore, a reduced astroglial reactivity could improve the survival of neurons. Indeed, GFAP null cells *in vitro* represent a better substrate for neuronal survival accompanied by neurite outgrowth improvement in comparison to GFAP positive astrocyte coculture (Menet et al., 2000). This phenomenon is observed *in vivo* only with double mutant GFAP and vimentin null mice. Five weeks after hemisection of the spinal cord, only the double mutant presents significant recovery of locomotor functions and reduced glial scars at the lesion site (Figure 12)(Menet et al., 2003).

Nonetheless, GFAP is an important factor shaping the CNS, especially in white matter and in the maintenance of myelination of neurons. In mutant GFAP null mice, 50% presented hydrocephalus associated with loss of white matter. Myelination pattern was impaired in those mice, with i.e., nonmyelinated axons in optic nerves, decreased myelin thickness in the spinal

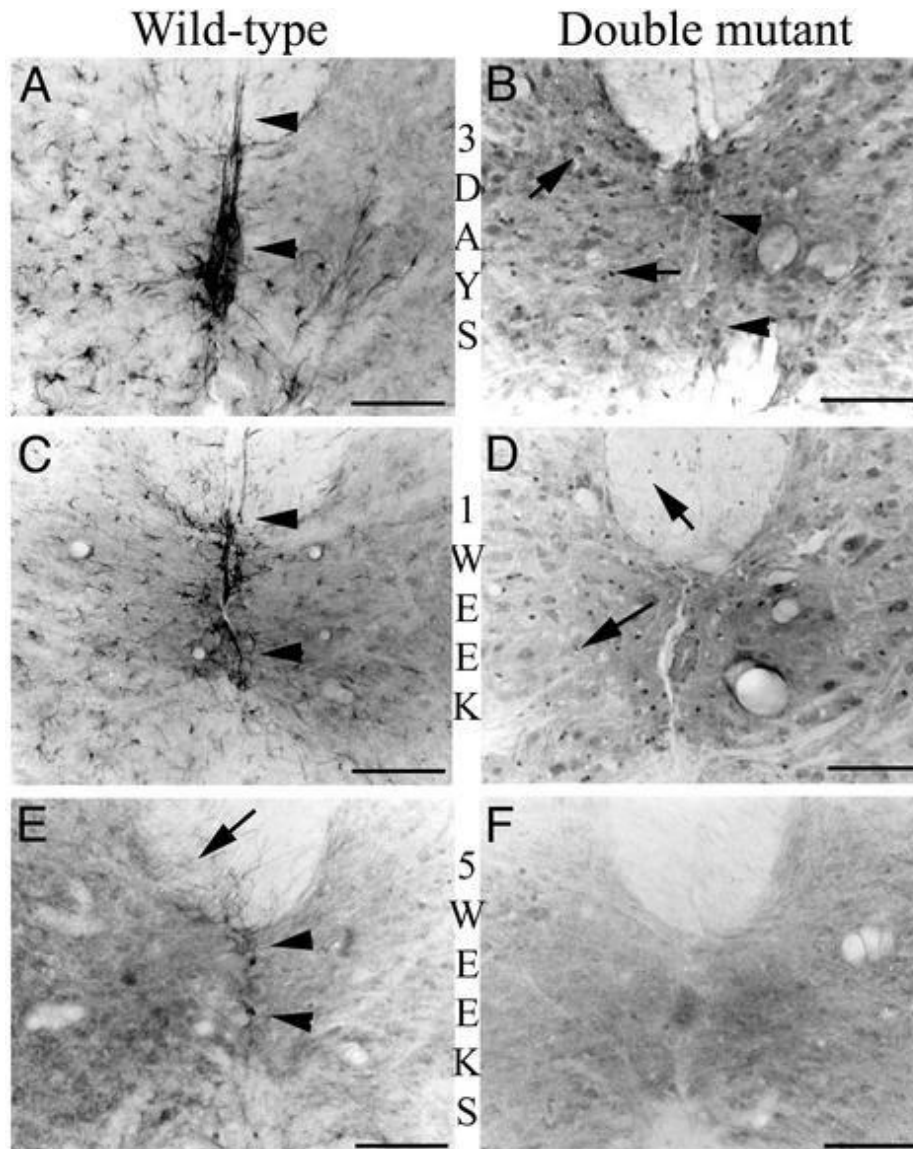
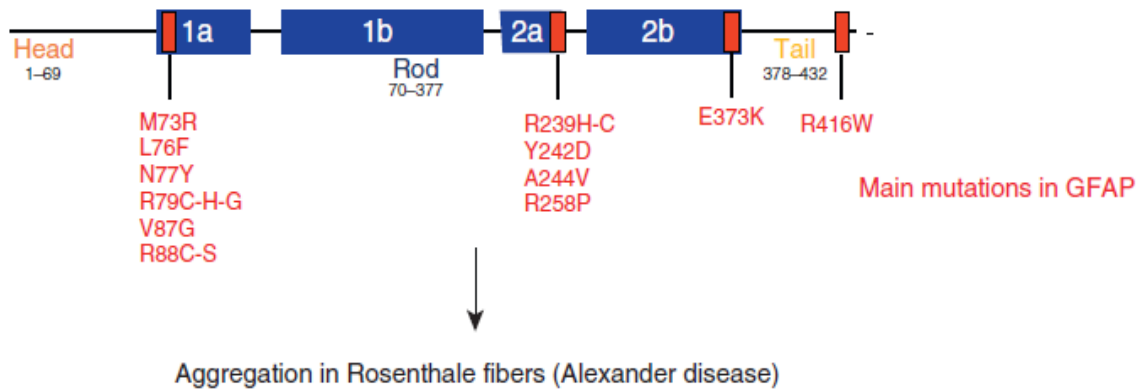


Figure 12 Astrocyte reactivity is reduced in space and time in double mutant GFAP-Vimentin mice. Here nestin was used to visualise glial scar. Three days after the lesion (A and B), nestin is confined to the lesioned side of the cord in a wild-type mouse (A), whereas a more diffuse pattern is observed in a double mutant mouse (B, arrows). We can observe the differential pattern expression in astrocytes and ependymal cells of a wild-type mouse (A, arrowheads) and of double mutant mice (B, arrows and arrowheads). One week after the lesion (C and D), nestin-IR is still present in the lesioned side of the cord of the wild-type mouse decorating the astrocytes processes and the ependymal cells (C, arrowheads), whereas it appears more restricted in a double mutant mouse, labeling only few perikarya of astrocytes in the gray and white lesioned matter of the cord (D, arrows). Five weeks after the lesion (E and F), nestin-positive cells are still present in the lesioned side of the cord of the wild-type mouse (E), predominantly in the white matter (arrow) and in the ependymal cells (arrowheads). In contrast, no nestin-IR cells are detected in a double mutant mouse (F). [Bar (A and B) _ 75 _m.] (image and caption: Menet et al, 2003)

cord and counterpart, actively myelinated oligodendrocytes. The vascularisation of the white matter was also poor, and the blood-brain barrier integrity, affected (Liedtke et al., 1996).

GFAP in disease. Undeniably, the main GFAP related disease exhibits an overexpression of GFAP due to heterozygous missense mutations on the GFAP gene, as displayed in figure 13, engendering a Rosenthal fibres accumulation which is proteinaceous aggregate found in astrocytes and the main cause of a neurodegenerative disorder called Alexander disease (Brenner et al., 2001; Sosunov et al., 2017). Twelve main mutations have been described and all appear *de novo* and lead to a dominant gain of function associated with the accumulation of GFAP and its incapability to polymerise and form normal network (R. Li et al., 2002).



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Figure 13 Mutation on the GFAP gene. The schematics show the main mutations (in red) observed in glial fibrillary acidic protein (GFAP) that give rise to Alexander disease (Dutour-Provenzano & Etienne-Manneville, 2021).

It is a very rare disorder, therefore the true prevalence is unknown. Since its first description, only 550 cases have been identified (Kuhn & Cascella, 2021). Alexander disease affects the normal development of the brain. Indeed, infants affected by this pathology exhibit a leukoencephalopathy accompanied by a macrocephaly, seizure and retardation in psychomotor development. These symptoms are the cause of a very short life expectancy, usually leading to death within 10 years (Brenner et al., 2001).

In conclusion, GFAP is a good marker that has allowed the characterisation of astrocytes *in vivo*. They are overexpressed in astrogliosis and are involved in the formation of glial scars. They are also overexpressed in disease, as seen in Alexander disease.

1-4-2-2. *Synemin*

Synemin is the least understood IFs expressed in astrocytes, probably due to the very few biochemical tools available to study it, in comparison to its counterparts. It was first identified coprecipitating with desmin in the chicken smooth muscle, letting researchers think that synemin was an IFs binding protein rather than an IF itself. The name *syn* (with) *nema* (filament) was then given from the Greek (Granger & Lazarides, 1980). Since then, proteomics studies

reveal that synemin has common structures with the other IFs, e.g., a rod domain of 310aa, but particularly with Nestin, which is the reasoning behind its placement in the type IV IFs group (Paul & Skalli, 2016). At a genomic level, it is known that synemin generates two different splice variants in humans: synemin α also called H and β also known as M or desmuslin. Synemin α is predominant in striated muscle, whereas both forms are present in smooth muscle (Figure 14) (Titeux et al., 2001). A third splice variant, L, is not found in humans, and is observed in rodents. The particularity of synemin structure in comparison with the other IFs is the very short head domain of only 10aa, and the 10 times longer tail domain of 1246aa for the α splice variant and 934aa for the β splice variant. The third splice variant is expressed in rodents is characterised by a shorter tail. The difference between the two human isoforms laying only in the long C-ter tail domain of the protein, it is highly probable that this difference gives rise to different subcellular localisation and binding partners (Russell, 2020). Certainly, in muscle cells, the two isoforms are localised differentially: the α form, primarily at the sarcolemma and the β form at the Z-disk (Lund et al., 2012). Synemin is mainly characterised in muscle cells, where its role has been the most studied. Synemin plays, however, an important role in astrocytes. In astrocytes, synemin is mainly expressed during development. In mouse embryo development, the different synemin isoforms are expressed at different stages.

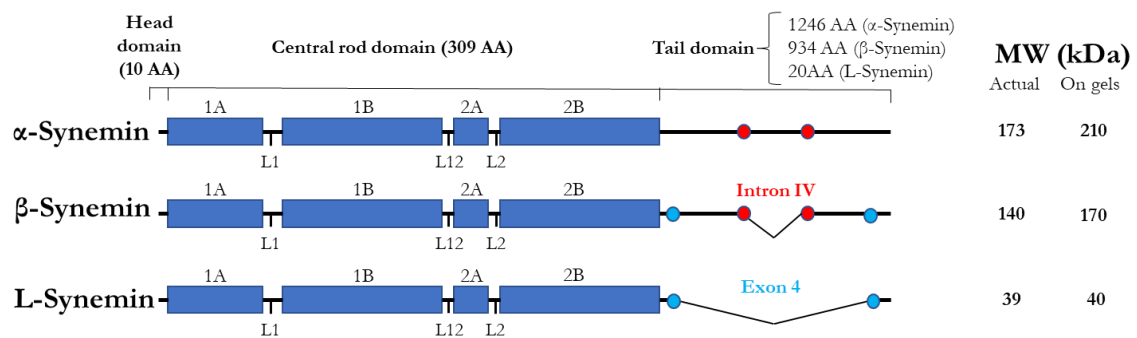


Figure 14 Structure of the three synemin isoforms. The proteins specificity is a very short head domain of about 10aa, and a long tail domain for the isoform α and β . The short head domain might be responsible for their incapacity to self-assemble into homopolymers. Similarly, to nestin, they are heavy proteins of around 173 actual molecular weight for the α -isoform, and 140 for the β one (adapted from Paul & Skalli, 2016).

There is first the expression of synemin β at E5 at the same time as vimentin and nestin. Synemin is first expressed in astroglial precursor cells that will give rise to other IFs expressing cells related to specific specialization. In astrocytes, synemin is first expressed with nestin followed quickly by vimentin and finally GFAP (Izmiryan et al., 2010). This dance of IFs seems to be important for cellular differentiation.

Synemin has multiple binding partners in muscle cells. Synemin binds to the actin network cross-linking protein α -actinin at costamers, a complex of protein related to FA (FA)

functionality in muscle (Figure 15). This interaction with α -actinin is interestingly observed at ruffled membranes in glioblastoma cell line U-373. This cellular domain is typically involved in cell motility (Jing et al., 2005; Pan et al., 2008). This might give a role of synemin in the increased motility in those tumoral cells. Synemin is also the only known IF that comes in direct contact with FA proteins. It has a known binding site on the C-ter tail domain with vinculin, and synemin is also able to bind talin, the molecular spring linking integrins and vinculin (Figure 15) (Bellin et al., 2001; Sun et al., 2008b; Uyama et al., 2006).

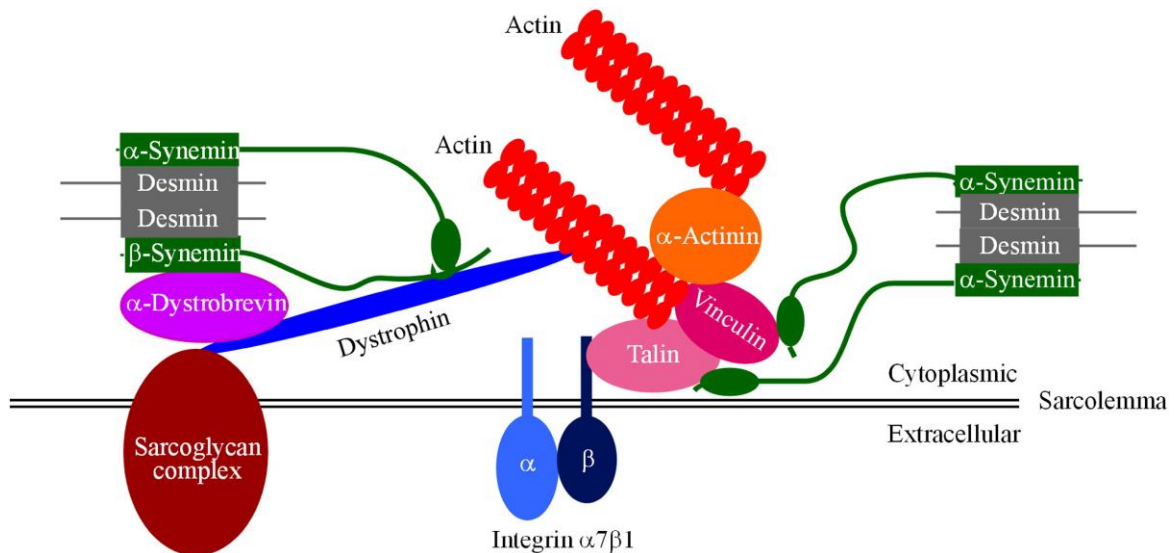


Figure 15 Interaction of synemin at costameres/ FA. Synemin is able to interact directly with FA complex protein such as vinculin and talin. The green ovals within the α -synemin tail domains represent the 312 amino acid inserts. Different α -synemin molecules are shown interacting with talin and vinculin, which attach the desmin/synemin heteropolymeric IFs to the costameres. The rod domains and tail domains of both α - and β -synemins are able to interact with α -dystrobrevin and dystrophin, respectively, which represents an alternative way of attaching the desmin/synemin heteropolymeric IFs to the costameres. (Image and caption: Sun et al., 2008)

Despite its unique mechanical properties and potential involvement in mechanosensing at FAs, synemin could sadly not be further studied in this thesis due to the lack of antibodies and constructs available in rat models.

1-4-2-3. Nestin

Nestin is a type IV IF, first discovered in the neuroepithelial progenitors. The name nestin was given because it was thought to be specific to the neuroepithelial cells (Lendahl et al., 1990). Nestin is primarily found in stem cells in the muscle within somites and the CNS, in the neuroectoderm. It was originally used to sort out stem cells from further advanced differentiated cells (Lendahl et al., 1990). The Spatio-temporal control of nestin expression might be thanked to the specific minimal promoter present in the 5' region and specific enhancer present in certain regions and during certain cellular processes such as embryonic patterning, migration and

proliferation (Michalczyk & Ziman, 2005). Only one variant has been identified in humans and, after synemin, is the largest IF in astrocytes with a molecular weight of 175-200kDa. The similarity with synemin is uncanny since they both share a similar structure comprising a small head domain and a long C-ter tail. With only 8aa, the head domain is preventing the self-polymerisation of the protein (Figure 16). The level of phosphorylation at the threonine residue at position 316 is tightly linked to the assembly and disassembly of the filament (Eriksson et al., 1992; Sahlgren et al., 2001). Indeed, at a low level of phosphorylation, the assembly process is engaged, whereas a three-fold increase of phosphorylation induces filament disassembly (Chou et al., 2003). Phosphorylation of nestin during mitosis is mediated by the cdc2 kinase (Sahlgren et al., 2001).

Human nestin 1q23.



Rat nestin 2q34



Mouse nestin 3 F1; 3 42.5 cM



▨ - untranslated region □ - coding region

■ - Region encoding helical rod domain ■ - Region encoding heptad repeat units

Figure 16 Nestin structure in humans, rats and mice. Similarly, to synemin, nestin possesses a short head domain and a long tail domain. The diagram shows the exon/intron structure of the nestin gene. The intron pattern is conserved between the human, rat and mouse nestin genes. The first three introns are in identical positions however the fourth intron has not been reported for the human gene (Michalczyk and Ziman, 2005).

Nestin function. By its specific stem cell expression, nestin is a marker for multi-potent neural stem cells. It is also active in several cellular processes happening in those cells. Nestin phosphorylation being associated with the disassembly of vimentin filament, particularly happening during mitosis, leads to an acceleration of cytoplasmic trafficking necessary to the assembly of filament, it is hypothesised that nestin expression helps facilitate rapid trafficking of IF precursors, as it is required in the development of nerves and muscle tissue (Lendahl, 1997).

Animal models. Studying nestin *in vivo* has proved to be rather difficult as the generation of a first nestin KO mouse engendered embryonic lethality with a penetrance of 90%. These mice

exhibit a lower number of NSC within the neuroepithelium of the developing neural tube, accompanied by an increased level of apoptosis (Park et al., 2010). Later on, two other nestin^{-/-} mice models were designed that could reach adulthood. The main phenotype exhibited by these mice are an aberrant distribution of acetylcholine receptors at the neuromuscular junction but indicated no big disturbance in the CNS (Mohseni et al., 2011).

Nestin in astrocytes. Nestin in astrocytes associates with vimentin or GFAP to form a filament. Moreover, vimentin increases nestin stability as nestin degrades more easily in vimentin null cells. The vimentin polymerisation is regulated by nestin, as an excess of nestin negatively affects the assembly of vimentin (Lindqvist et al., 2016). Nevertheless, nestin is not found in mature astrocytes and those interactions are only present in the developing astrocytes (Gilyarov, 2008). Nestin is, however, re-expressed in astrocytes during neuropathogenesis, such as CNS injury. After glial scarring, the expression of nestin in astrocytes at the site of the lesion is long-lasting, approximately 13month after trauma (Frisén et al., 1995).

Nestin in cancer. Nestin expression is involved in a variety of cancer, from breast cancer to prostate and hepatocellular carcinoma to gliomas, the latter being focused on later on in this chapter. In breast cancer, the expression of nestin is associated with higher tumour progression and metastasis (Nowak & Dziegiel, 2018). Mice exhibit lower metastatic rates when injected with nestin KO breast cancer cells, it is also associated with an increased survival rate in comparison with control cells. Cellular stiffness of the nestin^{-/-} cells being 1.5 fold increased, and rescued when re-expressing nestin, it was suggested that the metastatic capacity of nestin-expressing breast cancer cells was due to the cellular stiffness, given by nestin (Yamagishi et al., 2019). In the normal mammary gland, nestin is expressed only in the basal/myoepithelial layer. It is then overexpressed in adjacent cell types to participate in tumour development. In breast cancer, nestin is also found to participate in microvessel proliferation along with the proliferation marker Ki-67 and is featured in basal-like phenotype and more aggressive form of breast cancer (Krüger et al., 2013). This gives an active role of nestin in tumour angiogenesis (Figure 17) (Nowak & Dziegiel, 2018). Nestin expression being linked with metastasis and nestin ablation lead to inhibition of disease progression, the use of nestin-target therapy especially in the treatment of breast cancer brain metastases has a lot of potentials (Meisen et al., 2015).

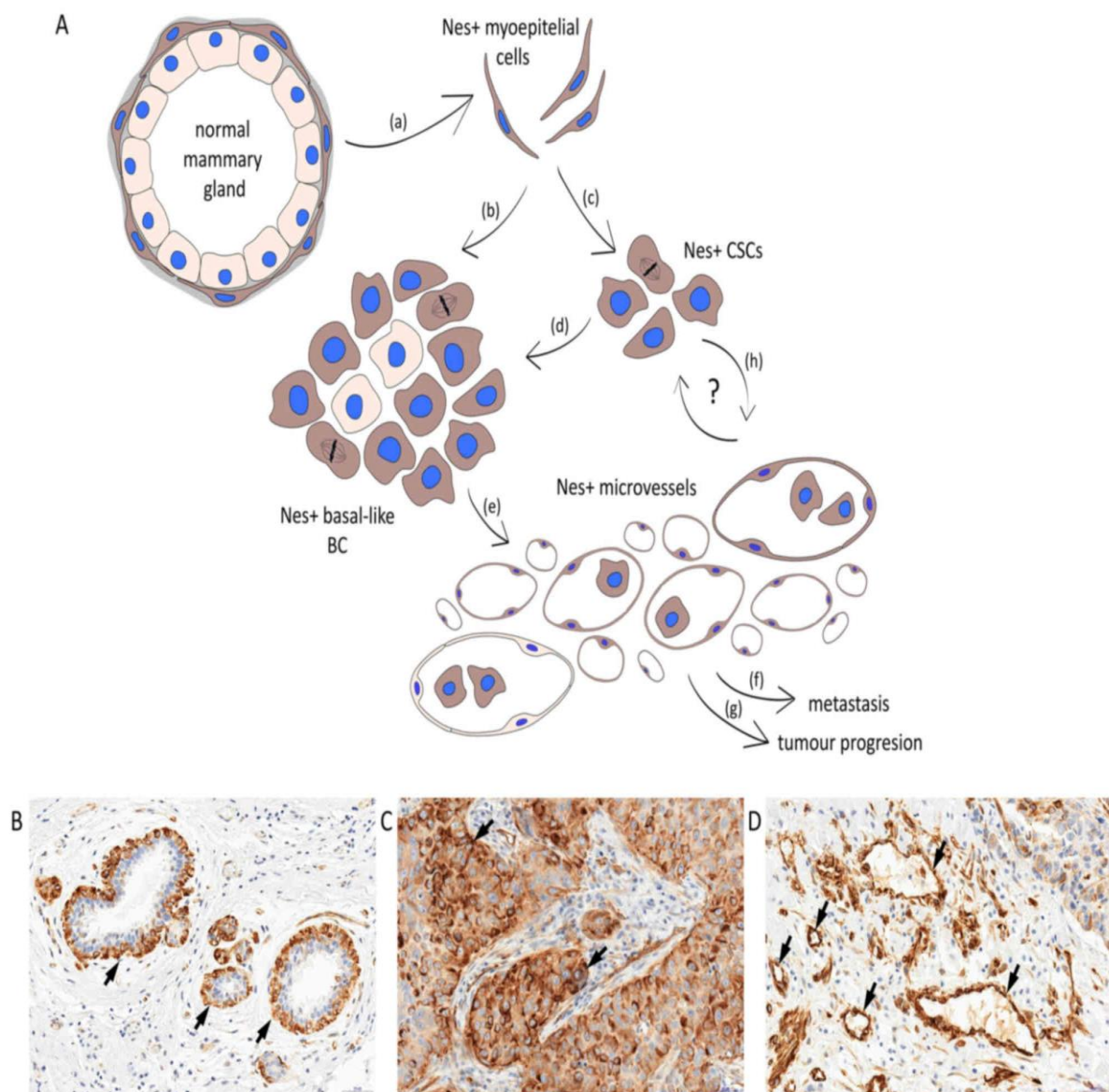


Figure 17 “Role of nestin in the pathogenesis of basal-like BC. (A) Nestin expression (brown) is detected in the basal/myoepithelial layer of the normal mammary gland. (a) Nestin+ myoepithelial cells give rise to (b) nestin+ basal-like tumours and (c) nestin+ CSCs that are able to (d) repopulate the tumour. Nestin expression is also observed in newly-formed tumour vessels. The number of nestin-expressing vessels correlates with (e) nestin expression in tumour cells, (f) metastasis and (g) tumour progression. (h) Some evidence indicates that cancer stem cells are also able to differentiate into endothelial cells and/or form vessel-like structures through a process termed vasculogenic mimicry. Immunohistochemically detected nestin expression in the basal/myoepithelial layer of (B) the normal mammary gland, (C) the basal-like BC and (D) the BC associated-vessels. Magnification, $\times 300$. Nes, nestin; BC, breast cancer; CSCs, cancer stem cells”. (Image and caption by Nowak & Dziegiel, 2018)

1-4-2-4. *Vimentin*

Vimentin is the major type of intermediate filament ubiquitously expressed in mesenchymal cells (Franke, *et al* 1978). This 57kDa protein from type III is the most studied IF as it is expressed in a very wide number of cells. Being from type III, vimentin can form homopolymers as well as assemble with a wide range of IFs isoforms.

Vimentin was initially isolated from mouse fibroblast culture. Hynes et al discovered a filamentous structure of 10nm by raising an antibody, that did not stain for either actin or MT, allowing to set it apart (Hynes & Destree, 1978). It was however later in the same year of 1978 that the protein was purified and named vimentin after the Latin *vimentum*, used to describe an array of flexible rods (Franke et al., 1978).

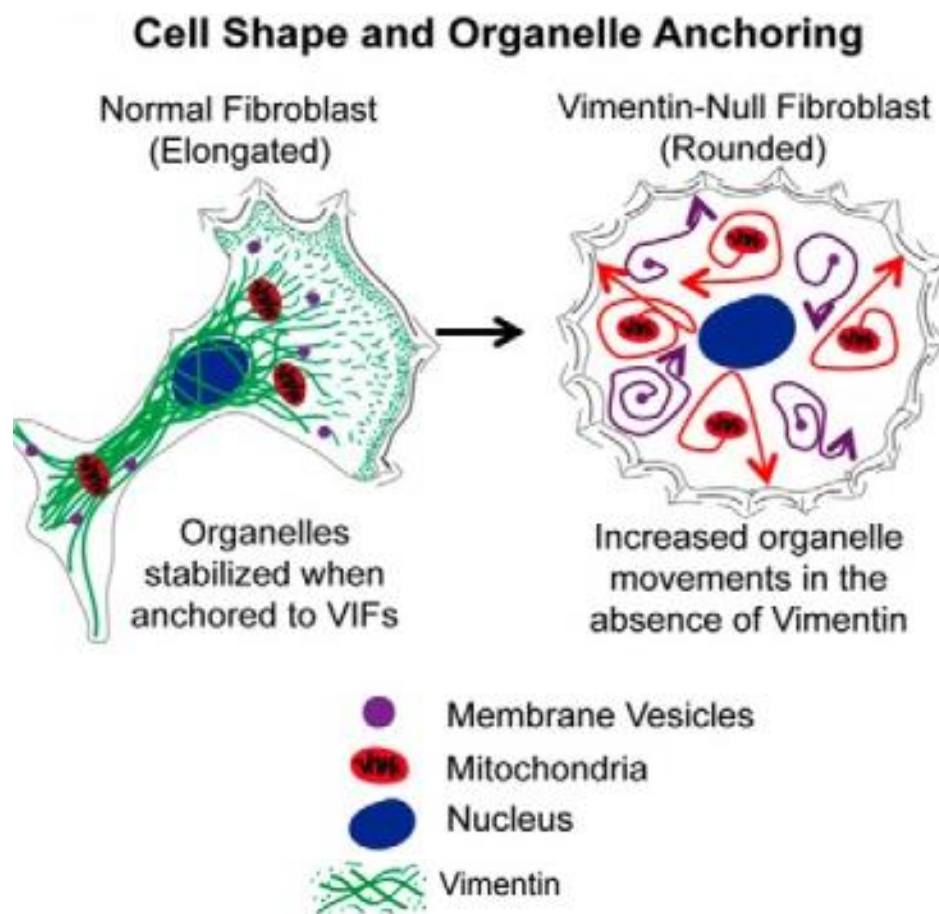


Figure 18 *Vimentin as limiter of movement in the fibroblast. The density of vimentin in the cytoplasm allows the anchoring of organelles in the cell such as the nucleus (in blue) or the mitochondria (in red) or membrane vesicles (in purple). The fibroblast cell shape is also affected (lowery et al, 2015).*

Vimentin functions. Vimentin is well studied to describe the mechanical importance of IFs in the cell. They have a first function as an organiser of the cellular space, anchoring different organelles and reducing their free random movement in the cytoplasm (Figure 18) (Guo et al., 2013). This particularity might be due to the intracellular cytoplasmic stiffening happening in

vimentin expressing cells. Indeed, fibroblast vimentin null cells have a two-fold reduction of cytoplasmic stiffness in comparison to the control (Guo et al., 2013; Mendez et al., 2014). Interactions between vimentin and the main organelles have been well characterised. Vimentin allows the positioning of organelles such as the Golgi through interaction with Golgi associated enzyme FTCD (for-mimino transferase cyclodeaminase) (Gao & Sztul, 2001). Vimentin is also involved in the positioning of the nucleus and its protection against compression (For more details see IV-3) (Dupin et al., 2011; Patteson, Vahabikashi, Pogoda, Adam, Goldman, et al., 2019). This protection is mediated by the cage-like structure that vimentin is forming around the nucleus, probably anchoring it via the LINC complex (See chapter IV-1), with the intermediary of plectin f1 (Keeling et al., 2017; Tusamda Wakhloo et al., 2020).

Animal models. Animal models were created to better understand the role of this IF. A complete knock-out (KO) of the vimentin mice model leads to viable embryos, healthy and able to reproduce. It is one of the reasons that vimentin was initially thought to not affect development, accompanied by the fact that MTs and actin filament are not compensating for the lack of vimentin (Colucci-Guyon et al., 1994). However, further investigation was made and a motor coordination impairment in vimentin KO mice was revealed. This phenotype proved to be induced by a defect in Bergmann glia and Purkinje cells. Indeed, cell bodies located at the vicinity of Purkinje cells are less differentiated and lacking defined nuclear and cytoplasmic structures (Colucci-Guyon et al., 1999). Observations about delayed fibroblast wound healing have been made, due to mechanically weak fibroblast in vimentin KO mice. They express difficulties in contracting a 3D-collagen network, an important step in cell migration (Eckes et al., 2000). The importance of vimentin *in vivo* in a great variety of tissue and metabolic changes is undeniable. We are here interested in the expression of vimentin in the brain and especially in astrocytes.

Vimentin in astrocytes. The expression of both Vimentin and GFAP in astrocytes is inversely correlated with age, as developing astrocytes present high vimentin and low GFAP relative expression and mature astrocyte, low Vimentin and high GFAP expression, corresponding to the time of myelination in the brain of the rat (Dahl, 1981). It is also interesting to note that vimentin is typically upregulated in astrocytes undergoing reactive astrogliosis (Pixley & de Vellis, 1984). Astrocytes devoid of Vimentin and GFAP, and subsequently of all IFs, present shorter processes showing a decrease in the hypertrophy after activation (Wilhelmsson et al., 2004). Reactive astrocytes express specific IFs depending on the region of the brain: indeed, in post-mortem healthy human brain, it was observed that vimentin immunoreactive astrocytes are more abundant in grey matter than white matter (O'Leary et al., 2020).

Vimentin is an indispensable IFs in astrocytes as Nestin and Synemin require vimentin to assemble into filaments, more than GFAP type III IFs, giving vimentin a better regulatory role in the structure of astrocytes (Pekny et al., 1995). A filament can possess all of the isoforms present in astrocytes: IFs act together as each protein can assemble along the same filaments (Figure 19).

Astrocyte main functions in a neurotrauma or disease state being the formation of glial scars. As described above, intermediate filament expression is upregulated in wound healing. Ablation of both vimentin and GFAP leads to a lower density scar accompanied by bleeding (Pekny et al., 1999). The double mutant (Vimentin and GFAP) KO leads to decrease astroglia reactivity linked with plastic sprouting of supraspinal axons.

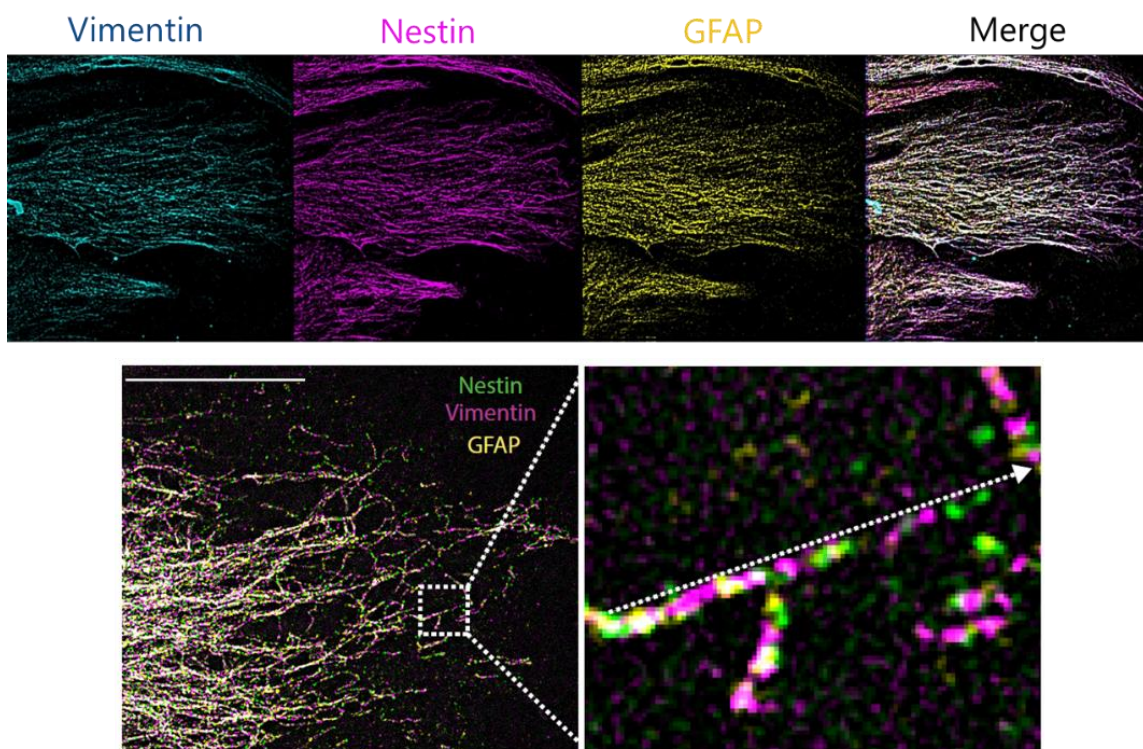


Figure 19 Isoforms of IFs assemble together to form heteropolymers and filaments. 3D structured illumination microscopy images of nestin (green), vimentin (magenta), and GFAP (yellow) immunostaining at the front of a migrating astrocyte. Kinesin depletion was used to decrease the density of IFs at the cell front, facilitating the visualization of single filaments. A higher magnification of the boxed region is shown in the middle (Leduc & Etienne-Manneville, 2017).

I-4-3. IFs in cancer: focus on Glioblastoma

A glance at what happens in healthy astrocytes is therefore important to better understand how malignancies such as glioblastoma rise and can thrive and spread in the brain. Understanding how the cell interacts with the many different extracellular signalling cues seems to be key in finding remedies, and personalised therapies. Intermediate filaments in astrocytes seem to be at

the centre of the cellular response to its environment and it is therefore important to study IFs, with higher magnification, in astrocytes.

Nestin in brain cancers. Nestin being a marker for poorly differentiated cells, and expressed when cells acquire the necessity to migrate and proliferate, it is used as a marker for a more defined grading of the tumour stages, especially tumour rising from progenitor cells. Logically, this protein is involved in cancer, especially in the different sorts of brain cancers, the CNS being one of the two regions expressing nestin. Intermediate filaments especially nestin co-expressed with vimentin are thought to control cell shape, morphology in GBM (Matsuda et al., 2013).

Nestin expression is tightly correlated with invasive phenotypes in astrocytoma cell lines (Rutka et al., 1999). The depletion of nestin in glioblastoma, the highest grade of gliomas, impairs their extension within the subventricular zone, acting through Notch and Kras signalling (Shih & Holland, 2006). Notch is a receptor in a simple signalling pathway indispensable for development and often implicated in the transformation of malignancy. Notch and its ligand are transmembrane proteins with an extracellular domain constituting epidermal growth factor (Bray, 2006). Notch in gliomas can directly act on nestin's regulatory region to activate its expression (Shih & Holland, 2006).

GFAP in brain tumours. GFAP expression levels have been debated to be an indicator of malignancy level for gliomas. However, the serum level of GFAP could well serve as a diagnostic tool in glioblastomas. In a study driven by Jung *et al*, GFAP was found in the serum of glioblastoma multiform (GBM) patients before tumour resection at a median of 0.18µg/L, compared to the absence in non-GBM patients and healthy controls. Levels of GFAP in the serum also correlate with tumour size and necrosis, making GFAP a good potential biomarker for GBM (Jung et al., 2007; Tichy et al., 2016).

Vimentin in brain tumours. As for Nestin and GFAP, an increase in vimentin expression is observed in gliomas and GBM. High vimentin expression in glioblastoma is associated with a lower survival rate and lower progression-free survival (J. Zhao et al., 2018). Vimentin therefore can be used as a prognostic outcome for the patients. Interestingly, glioblastoma patients expressing low vimentin are more susceptible to the therapy temozolomide, commonly used in glioblastoma treatment (L. Lin et al., 2016). Vimentin is also involved in migration as the downregulation of vimentin in GBM cells, significantly impairs their migration (Nowicki et al., 2019).

Overall, IFs are very important in the development, progression and migration of gliomas and glioblastomas. They are used for grade differentiation, prognostic marker, and could be the next target for treatment.



Astrocytes have a specific expression of IFs: vimentin, nestin, GFAP and synemin. GFAP is almost exclusively expressed in astrocytes, it is used as a marker. Nestin and vimentin are expressed in developing astrocyte whereas GFAP is expressed in mature astrocytes.

I-5. Cytoskeletal interactions:

I-5-1. Interactions between cytoskeletal networks

The cytoskeleton forms within the cytoplasm an intertwined and highly dynamic network of filaments. They are interacting in several ways, indirectly, thanks to cytoskeleton cross-linker, and directly. There is indeed a dynamic and active crosstalk between the different components of the cytoskeleton. This has been well studied for the crosstalk between actin and MT. This crosstalk is essential for a lot of cell processes such as cytokinesis and migration. As they share regulators and crosslinking proteins such as EB1 protein interacting with the plus tip of MT, they can act as a guidance system for MT growth and give dynamic links between the two networks (Alberico et al., 2016). More interactions between MT and actin are thoroughly explained in this review: (Dogterom & Koenderink, 2019).

IFs exert as well a regulatory role through intricate crosstalk between actin and MTs. IFs interact with the other cytoskeleton components mainly through crosslinkers such as adenomatous polyposis coli (APC) or plectin. The effect of that interaction is yet to be clearly understood. Vimentin, for example, controls the formation of actin stress fibres by inhibiting RhoA through interaction with guanine exchange factor GEF-H1 (Jiu et al., 2017). Hence, an increase of traction forces is observed in the U2OS osteosarcoma line in absence of vimentin. Vimentin associate with MT and are aligned in polarised cells (Sakamoto et al., 2013). MTs that are directly associated with vimentin are more resistant to MT depolymerization drugs nocodazole. After treatment, MTs grow along the vimentin filament as a template for its network (Gan et al., 2016). This provides evidence that crosstalk between IFs and the other two networks is of utmost importance for cellular homeostasis.

I-5-2. Transport of IFs in the cell: interaction with cellular protein motors

There have been two different kinds of transport of IFs within the cell identified. Both kinds rely on the interaction with the other cytoskeleton proteins: actin and MT.

MT dependent transport of IF proteins. The first type depicts a rapid, discontinuous and bidirectional movement. As MT depolymerisation drug nocodazole disrupts this type of transport, it was characterised as MT dependant (Francis et al., 2005; C. L. Ho et al., 1998).

IFs proteins or ULFs are seen as cargoes by the motor proteins for transportation along actin or MTs (Figure 20). Determination of the interaction between the MT molecular motor kinesin at the plus tip of the MT was pivotal to understand this type of movement in the cell (Gyoeva & Gelfand, 1991). The interaction with IFs and the kinesin is specific to the tail region of the heavy and light chain of the kinesin. The interaction also requires the detyrosination of MTs, acting as a signal for the recruitment of IFs to the MT, via the binding of kinesin (Kreitzer et al., 1999; Liao & Gundersen, 1998). It was shown a few years later that IFs also bind the second

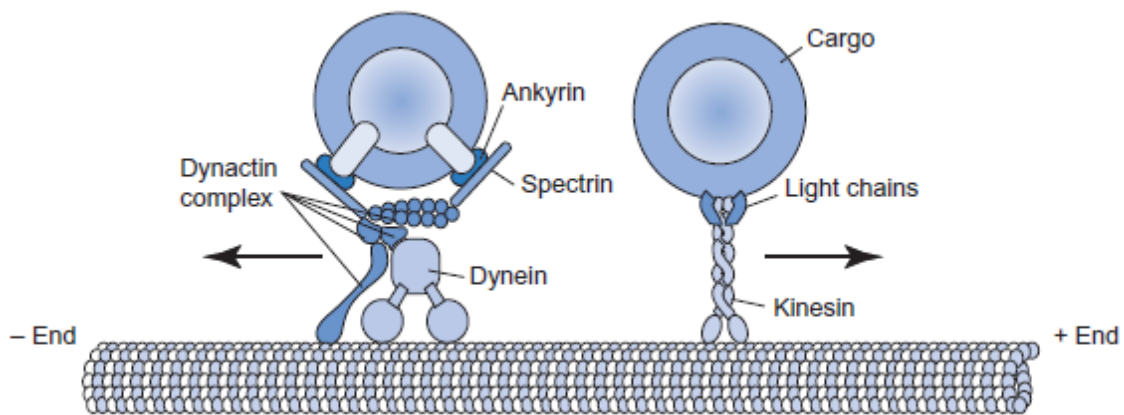


Figure 20 Transportation of cargoes along microtubule. Here cargoes can represent IF subunits being transported to be incorporated within the filament. Commonly, cargoes are moving from the -end to the +end attaching to the light chains of kinesins and on the opposite direction with the dynein complex composed of dynactin with spectrins and anchored thanks to ankyrin (Shah et al., 2020).

MT-based motor protein dynein at the minus end. This was demonstrated for vimentin, neurofilaments and peripherin. The medium subunit of the neurofilament bind directly to the dynein intermediate chain (Helfand et al., 2002, 2003; Shah et al., 2000). Binding to either dynein or kinesin is determining the direction of movement of IFs subunits (Figure 20). This explains the bidirectional movement observed early on. The IFs cargo can be attached to both dynein and kinesin at the same time. They will then start a “tug-of-war” pulling on the cargo. Mathematical modelling seems to predict that the elasticity of the cargo and the number of motor binding sites will affect the directionality of the IF cargo (Dallon et al., 2019; Ikuta et al., 2014). A study about peripherin IF motility showed that peripherin can be co-translated locally

for a fast local reorganisation of the network. Indeed, peripherin mRNA containing ribonucleoproteins have been observed moving along MTs. Upon the end of the movement, the translation is induced in a process of dynamic co-translation (Chang et al., 2006).

Actin dependent transport of IFs. The second kind of transportation of IFs in the cell is described as slow, continuous and unidirectional. It is linked to actin flow. Where extensive researches have been done on the transport of IFs by MT and interaction with MT motor protein, very little research on this second type of transport have been done. This type of transport starts at the periphery of the cell and is directed towards the cell centre. It has been studied for keratin network organisation. After disruption of the actin network, keratin subunits polymerise at the plasma membrane but remains there. Keratin starts being generated at FAs and assembles along actin filament to be integrated into the network (Figure 21) (Kölsch et al., 2009; Leube et al., 2017).

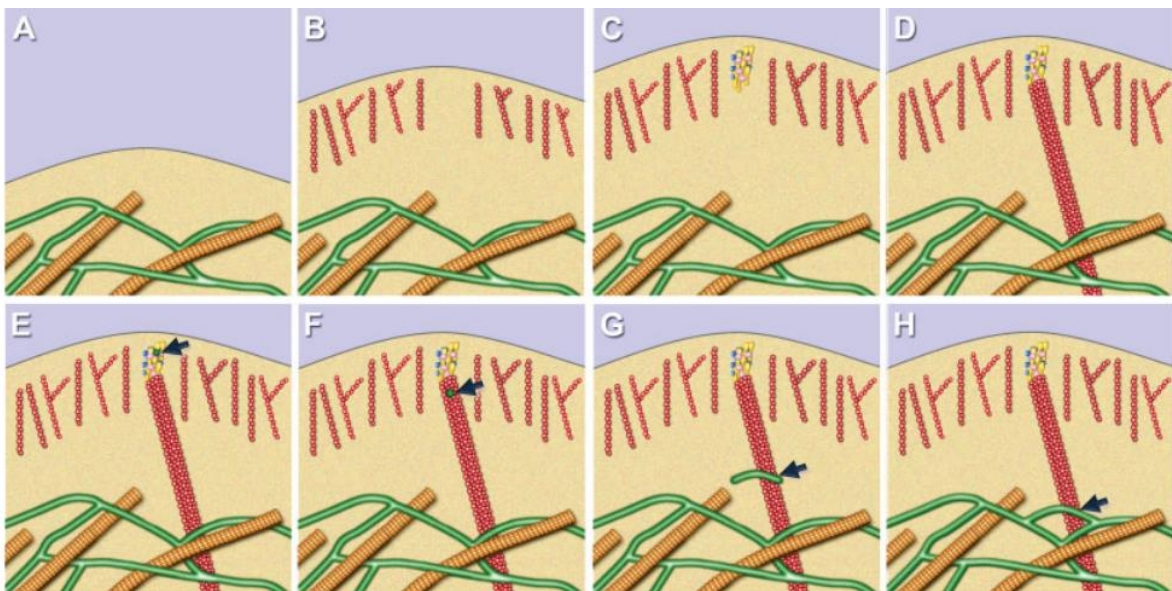


Figure 21 Transport of keratin subunit along actin filament. Schematic representation of sequential steps of KF formation and transport in the cell periphery. The cell front is shown together with the peripheral KF network (green) and microtubules (yellow) in (A). A lamellipodium develops by peripheral actin filament polymerisation (red; B). FAs are formed as large multiprotein complexes beneath the Collapse (Kölsch et al., 2009).

In certain cell types, IFs are associated with only one type of transport. For example, Neurofilaments are transported along the axon in a MT-dependent manner only. They do not require actin. The opposite is observed in keratin expressing cells, where MT network disturbance do not affect the transportation of the keratin (Kölsch et al., 2009). However, the transportation of fully formed keratin filament can use the MT network via kinesin-1, when actin transportation is impaired (Robert et al., 2019).

This shows that the interaction of IFs with actin and MT, especially during assembly of IFs is crucial. Nonetheless, fully formed networks of the three cytoskeleton proteins interact in different cellular processes to maintain cell homeostasis.

I-5-3. A fully formed network of IFs interacts with actin and MT

The structural and mechanical functions of IFs are reinforced by their tight connection and crosstalk with the actin and MT networks. This is perfectly illustrated in muscle fibres, where desmin filaments connect the Z-discs to the plasma membrane, mitochondria and nuclei to increase the mechanical resilience of muscle cells (Conover & Gregorio, 2011; Mermelstein et al., 2006; Milner et al., 2000). In addition to a possible direct interaction with actin microfilaments, a wide variety of cytoskeletal crosslinkers connect IFs to actin and MTs: these include plectins, alphaB-crystallin, fimbrin, filamin A, adenomatous polyposis coli (APC), as well as several cytoskeletal motors (Djabali et al., 1997; Karashima et al., 2012; Leduc & Etienne-Manneville, 2017; Sakamoto et al., 2013). The impact of IFs not only is a result of their physical interactions with actin and MTs but also involves their function in key signalling pathways controlling, for instance, RhoA activity and cell contractility (Jiu et al., 2017). IFs influence the dynamics and organization of the MT and actin networks. Accumulating evidence points to IFs as major regulators of actin stress fibres and actomyosin-mediated forces, although the effects vary with cell type, possibly as a consequence of differences in IF composition (De Pascalis et al., 2018; Jiu et al., 2017). Although no major change in the global organization of the MT network has been observed in IF-depleted cells, IFs can modulate the dynamics of MTs. Vimentin IFs interact with MTs and serve as a template to subtly direct MT growth (Gan et al., 2016). MTs that are directly associated with vimentin are also more resistant to MT-depolymerizing drugs like nocodazole (Gan et al., 2016). Whether all types of IF have the same effect on the other cytoskeletal networks awaits further investigation. However, it is already clear that keratin and vimentin networks behave very differently concerning MTs, as MT depolymerization induces the perinuclear collapse of the vimentin IF network without affecting keratin IF organization (Kölsch et al., 2009). Overall, it is likely that IF composition or post-translational modifications influence cell contractility and motile behaviour and may be key to the adaptation of various cell types to the specific, and possibly evolving, mechanical properties of their environment (Dutour-Provenzano & Etienne-Manneville, 2021; van Bodegraven & Etienne-Manneville, 2021).

Interactions with adhesive structures. The scaffolding structure formed by cytoplasmic IFs, together with the other cytoskeletal networks, is connected to the extracellular microenvironment. IFs interact with, are regulated by and also influence cell-cell and cell-matrix adhesive structures (Jones et al., 2017). This was initially illustrated by the association of keratin

filaments with desmosomes via desmoplakin and with hemidesmosomes via bullous pemphigoid antigens 1 and 2 (BPAG1 and 2) (Fuchs & Wiche, 2013). Keratin IFs stabilize hemidesmosomes and desmosomes, which are essential for the cohesion of epithelial tissues. The stabilizing role of IFs on cell adhesions is not limited to epithelial cells. IFs are involved in the stability of gap junctions in cardiomyocytes and of adherens junctions in endothelial cells. IFs also associate with FAs in many cell types, including fibroblasts, endothelial cells and astrocytes (Leube et al., 2015). The organization of IFs at FAs and the exact molecular link that connects IFs to integrins are not entirely clear and likely vary depending on the IF composition and on the integrin involved. One specific IF protein, synemin, directly interacts with several FA proteins, such as talin, vinculin and zyxin (Russell, 2020; Sun, 2008). In the absence of synemin, plectin (more specifically plectin 1f) is involved in the association of vimentin and desmin with integrins (Sun et al., 2008a). IFs tend to stabilize or reinforce FAs in immobile cells. However, the molecular mechanisms physically and functionally bridging IFs to FAs need to be further investigated, focusing on the specific relationship of each IF protein with various integrins and FA proteins (Dutour-Provenzano & Etienne-Manneville, 2021).



Note:

IF proteins are transported towards the nucleus by retrograde flow of actin, where they tend to accumulate.

I-6. Key messages

- A. The three different cytoskeleton component possesses different organisation and structures within the cell and tissue. Where actin and MT have a polar and energy-dependent assembly, IFs are apolar and have a very specific mode of assembly
 - B. IF proteins are encoded by a super family of at least 75 genes. There are two distinct types: cytosolic and nuclear IFs. Both have a similar structure composed of a head domain, a rod domain of constant length throughout all isoforms and a tail domain. The difference between cytosolic and nuclear mainly lies in the NLS sequence and CAAX box on the tail domain allowing them to enter the nucleus.
 - C. cIF monomers assemble into dimers in a coiled-coil fashion, the formation of tetramers in an anti-parallel manner. Tetramers assemble into ULF and end to end into filaments.
 - D. The differences between IF isoforms lie within their head and tail domain. They are classified into six different categories, depending on their structures but most predominantly for their tissue-specific localisation.
 - E. In our model, astrocytes are present five isoforms of IFs: type III vimentin and GFAP and type IV nestin and synemin. They are found to be upregulated in glioblastoma.
 - F. The cytoskeleton is intrinsically interacting with numerous cellular processes. In the case of IFs, interaction with MT and actin are essential for their transport and assembly. They also regulate one another in several cellular activities such as migration.
- ➔ The diversity of IF proteins and their cell type specificity have made the study of IF functions more difficult. Although it is now clear that they interact with the other cytoskeletal components, and thereby contribute to numerous cellular processes, their exact roles still remain elusive. However, their dynamic properties point to their role in cell mechanics.

The mechanical functions of the cytoskeletal network

II. The mechanical functions of the cytoskeletal network

The three components of the cytoskeleton exhibit different assembly mechanisms leading to different physical properties. MTs and Actin polymerize in a polarized manner and both require energy to form a filament. In contrast to the polarized, energy-driven assembly of MTs and actin, IFs are apolar filaments allowing them to be oriented and elongated in both directions. These specific mechanical properties that are used by the cell for different functions. To understand better, the specific contributions of cytoskeletal networks are described below.

II-1. Actin and actomyosin

To characterise and study closely actin physical properties, *in vitro* studies have long been performed, growing the polymer directly on glass. Solutions need to be implemented with ATP, due to the energy-dependent growth of actin. Disregarding their rigidities, actin mechanical properties, measured *in vitro* by their persistence length, are deeply influenced by thermal regulation. This leads to bending fluctuation of the filament (Gardel et al., 2004). However, *in vivo*, polymers of actin are usually not bare but actin crosslinking protein forms bridges between the filaments. These proteins are called Actin-Binding Proteins or ABP. The concentration of ABP and the number of bindings on a filament sensibly affect its elasticity.

Actomyosin: Molecular motors such as myosin motors cross-link the filament of actin and myosin creates tension on the filament by stiffening the network (Koenderink et al., 2009). These actin motor proteins are mainly responsible for the internal tension of the network and its flexibility. The association of actin with myosin forms the actomyosin network and contractile stress fibres. The general mechanism of contractility of the actomyosin networks starts with myosin II heads that attach and detach the actin filament allowing a bi-directional axial sliding leading to the contractility of actin bundles. This process is ATP-dependent as myosin II need ATP cleavage to “walk” on actin (Senju & Miyata, 2009). Actin being anchored at the cell membrane through FAs complexes, the actomyosin contractility leads to the contraction of the cell (Colombelli et al., 2009). Stress fibres are motors of cell shape changes and movement of organelles within the cell and movement of the cell itself. As seen previously (see chapter 1), stress fibres are put in different categories, depending on their structure and localisation. Even if not all stress fibres are contractile, such as dorsal fibres, they all play a role in the contractility of the cell. Indeed, even if dorsal fibres are not contractile, they are associated with FAs (see chapter 3). Transverse arc can generate forces transmitted through the dorsal fibres thanks to their anchorage at FAs. Ventral and longitudinal stress fibres generate the most important contractile forces. To study the biomechanical properties of stress fibres, researcher came up with a way to extract them and study them in a cell free system (Kassianidou & Kumar, 2015). However, since stress fibres are an association of a myriad of binding proteins and

motors, some studies have found a way to study the properties of stress fibres without changing their environment by de-roofing fibroblast, leaving behind only FAs and associated fibres (Katoh et al., 1998). Both isolated and cell roof free methods allow a better understanding of the actin mechanical properties. Decreasing contractile ability with blebbistatin drug leads to the decrease in stiffness, in contrast, increasing contractility with calyculin A shows increases stress fibres stiffness (Lu et al., 2008). This suggests that the capacity of contraction of the actomyosin networks accounts for its mechanical stiffness in the cell.

Cortical actin: cortical actin constitute a densely packed polymerised network of actin filaments associated with actin binding proteins located underneath the plasma membrane and contribute greatly to the mechanical rigidity of the cells (Gilden & Krummel, 2010). Changes in the local actin cortex leads to tension gradient and local contraction of the bundles leading to deformation of the membrane. This modulation of the cortical actin is key in several processes such as cell division and migration (Chugh & Paluch, 2018).

Filopodia: Filament of actin and stress fiber polymerisation account for the formation of filopodia and lamellipodia. Filopodia are protusion found at the leading edge of migrating cells. They are formed by actin polymerising and assembling into bundle against the membrane in the migrating direction, pushing and creating a protusion. There is a great turnover of actin subunit added to the tip of the filopodium, then moved away as part of the filament lattice and released at the rear (Svitkina, 2018).

II-2. Microtubules

MTs are the largest filaments and display specific mechanical properties. Several ways are used to determine the mechanical properties of MTs: an experimental *in vitro* approach, using laser and optical traps and a dry lab approach using atomic and molecular models of predictions (Liew et al., 2015).

To study the mechanical properties of MT *in vitro*, studies using optical tweezer have shown how MTs can be deflected when a few piconewton (pN) forces were applied. The MT started to bend at a critical load of 1.5pN. Behind the critical force, the beads escaped the trap and MT goes back exactly to its origin stage (Kurachi et al., 1995). This accounts for their high stiffness. Stiffening of MTs accounts for more rigid network and make the cell more resistant to compressive stress (Matus, 1994).

Post-translational modification especially affects the mechanical properties of MTs. The principal PTM observed in differences of mechanical properties of MT is acetylation. It is marking stable and long-lived MT. Using FRET-based assays reporting on lateral interaction

between the protofilaments, acetylated MT resist higher mechanical forces. Using microfluidic assays, acetylation at the α K40 site was demonstrated to increase the resilience and flexibility of long-lived MTs (Portran et al., 2017; Xu et al., 2017). Mechanical anisotropy could be determined using the tip of an atomic force microscope to elastically deform a single MT anchored on a substrate tailored by electron beam lithography. Young's modulus and shear moduli could thus be measured. The bending modulus was estimated at 20 ± 10 MPa at 20°C (Kis et al., 2002). However, equipment needed to measure need extreme accuracy and it might not reflect the properties of single MTs. Researchers have come up with computational ways using atomistic simulation and mechanical modelling for more accurate values (Wells & Aksimentiev, 2010).

The role of MTs in cell mechanics remains fairly elusive. However, interplay between actin and MTs is important in several cellular processes such as cell migration and especially at the protruding edge. As seen earlier, lamellipodia contain a dense network of actin but also a few array of MTs. Inhibition of actin by cytochalasin D, which inhibit actin polymerisation in some cell type such as neuron and astrocytes do not inhibit protrusions, but instead promote neurite extension and membrane protrusion in astrocytes (Baorto et al., 1992; Bradke & Dotti, 1999). In this same cells, inhibition of MTs or kinesin motor activity leads to a cease of lamellipodial activity and protrusion (Ballestrem et al., 2000; Etienne-Manneville, 2004; Y.-L. Hu et al., 2002).

The association of molecular motors to MTs can modulate the mechanical properties of MT. Indeed, the Young modulus of MT has decreased anti-proportionately with the increase of kinesin interaction. Kinesin softens MTs and can therefore modulate their rigidity (Kabir et al., 2014). Association of MTs and molecular motors is important in cell mechanics and especially in cell division. Forces are needed to segregate the chromosomes provided by the formation of MTs into spindles. The active forces are generated by MTs assembly and disassembly cycles and by molecular motors. Addition of GTP bound and lost of GDP-bound subunits release energy transformed in mechanical work. The MTs filament themselves can also produce forces. Molecular motors involved comprises kinesin-5 and-8, and dynein. The directional movement of the motors along the MTs allowed by the hydrolysis of ATP create forces that induces the movement of cargoes, such as chromosomes. Passive forces are also important and comprise elastic forces, viscous drags, or frictional resistance such as the stretching applied between sister kinetochores (Forth & Kapoor, 2017b).

II-3. Intermediate filaments

IFs are more elastic and flexible than actin and MTs. Where actin and MTs are stiff structure with a high persistence length, from 7 to $22\mu\text{m}$ for actin to a few mm for MTs, IFs display

flexible structure with a persistence length ranging from 0.2 for Neurofilaments to 2.1 μm for Vimentin (Block et al., 2015b). They are, therefore, less susceptible to breakage. It is therefore odd that they are the least studied in the question of mechanics of the cell, with actin being by far ahead. The following part will hopefully convince you of the impressive mechanical characteristic of IFs.

II-3-1. Physical and mechanical properties of IFs

Due to their unique mechanical properties, IFs can take on the load as mechanical support of the cells. They are structuring the cells creating a scaffold anchored to the nucleus and throughout the cytoplasm (Patteson, Vahabikashi, Pogoda, Adam, Goldman, et al., 2019). Different ways are used to study the mechanical properties of IFs (Lowery et al., 2015). They can be reconstructed *in vitro*, studied within the cell or in tissues.

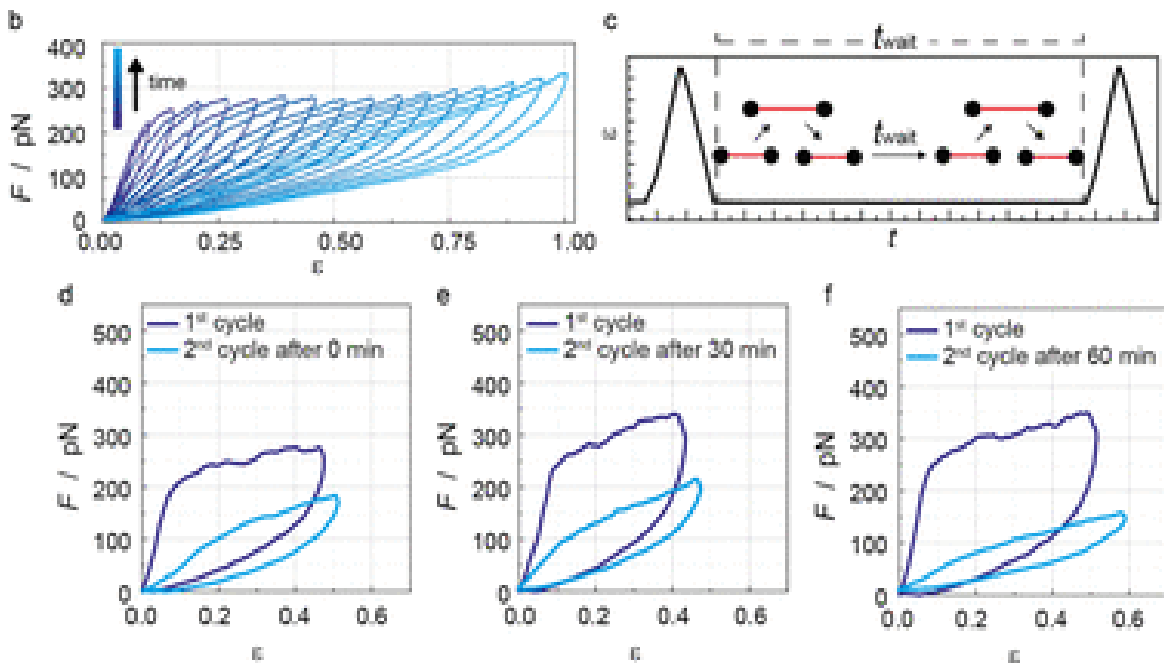


Figure 22 Stretching and elastic properties of vimentin Vimentin filament stretched to increasing distances with each cycle), (c) Sketch of the experimental protocol for stretching cycles, including waiting time (t_{wait}). (d–f) Examples for force–strain data from experiments with different waiting times ((d) $t_{\text{wait}} = 0$, (e) $t_{\text{wait}} = 30 \text{ min}$, and (f) $t_{\text{wait}} = 60 \text{ min}$). IFs are sensitive to repeated stretching. They do not recover completely by themselves and need introduction of crosslinker (Forsting et al, 2019).

IFs are grown in solution or coverslips where their mechanical properties can be closely assessed. IFs display a flexible structure, assembled by 45 nm long coiled-coil dimers with a persistence length ranging from 0.2 μm for Neurofilaments to 2.1 μm for Vimentin (Block et al., 2015a). The flexibility of the filament is depending on their specific type of assembly involving an axial sliding upon force application allowing the filament to be stretched (Mücke et al., 2004). This ability to stretch and deform under tension comes from their coiled-coil alpha-helical rod domains that can unfold and form β -sheets. IFs proteins come in contact through different

points of assembly along the rod domain to form a filament (C.-H. Lee et al., 2020). Recent studies have shown that the stretching of the filament is only partially reversible, meaning that when subjected to the second cycle of stretching, the filament displays lower extendibility (Figure 22). The filament once stretched in β -sheet does not refold when relaxed, thus exerting lower forces. The reversibility can be achieved with the introduction of a crosslinker, preventing the filament to enter the β -sheet state (Forsting et al., 2019). Inter-filament crosslinking also gives the filaments their astonishing elasticity. In order for the filament to stay in a stretched condition, energy is required. This energy is given by the hydrophobic and electrostatic inter-filament bounds. IFs are extremely elastic *in vitro* giving them great importance in cell integrity.

The remarkable mechanical properties of IFs are also essential within the cells. The cells are constantly afflicted by mechanical forces with, for example, membrane tension allowing the cells to change shape under external forces (Pontes et al., 2017). Therefore, they require a scaffolding structure to maintain their integrity. In keratinocytes, a strain stiffening is observed upon AFM (Atomic Force Microscopy) indentation measurement on junctions and lamellae in comparison with Keratin KO, suggesting that Keratin protects cell structures and may play a role in transducing forces from the junction to the cell body (Ahrens et al., 2019). In the same cell line, using AFM and magnetic tweezers, a significant softening of the cell is observed when the entire Keratin gene cluster is missing. This reduces cell viscosity and thus increases the cell deformability, a phenotype that can be rescued upon reintroduction of Keratin K5 and K14 (Ramms et al., 2013). In a study using unanchored MSCs, the authors stipulate, that cells with higher Vimentin expression exhibit a higher capability to deform upon strain application on different substrate rigidities (Sharma et al., 2017). This indicates the role of Vimentin as a cell shaper under external tension, especially during mesenchymal stem cell transition when the cell has to squeeze through the epithelium cells. The role of Vimentin as a protector of the cell is also described in MEFs cells where Vimentin protects the nucleus and regulate its shape, avoiding nucleus rupture when the cell is migrating through confined spaces. The nucleus of Vim KO MEFs is more susceptible to leakage, as seen using transwell migration assays (Patteson, Vahabikashi, Pogoda, Adam, Goldman, et al., 2019). Lamins also participate in the upholding of nucleus integrity. Their elastic capabilities are crucial during cell compression. The rod domain of Lamins can vary from 40 nm to the originally measured 52 nm. This size reduction might be due to the sliding of dimer creating an increase in rod overlap or a simple shortening of the rod by 15 to 20% (Makarov et al., 2019). Lamins are the only IFs that are positively charged right after the rod domain, creating an electrostatic interaction facilitating the assembly of dimers. These interactions and the presence of cross-linkers allow the lamin to get

stretched upon force application and to go back to their initial shape once the force is removed (Makarov et al., 2019).

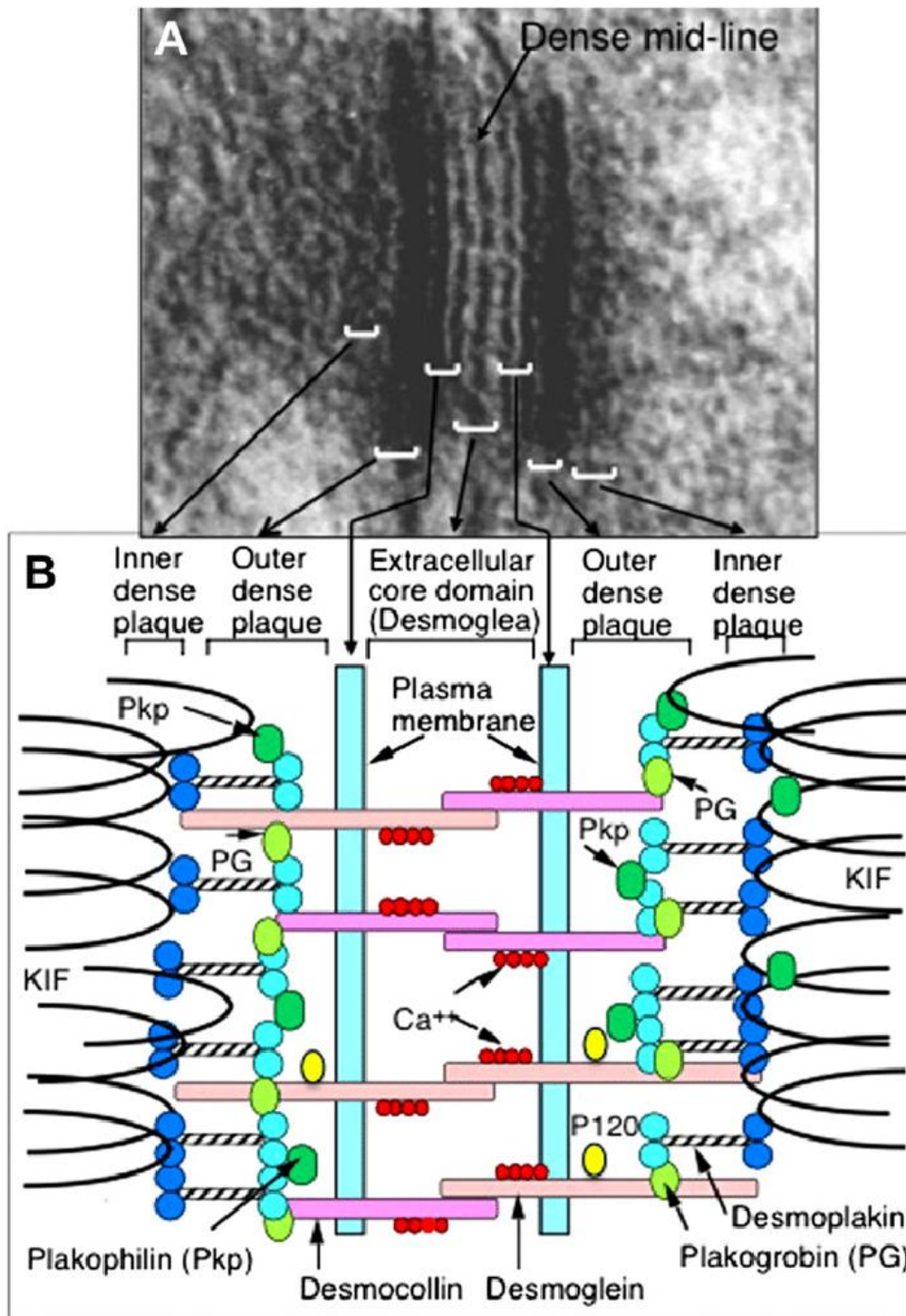


Figure 23 Desmosome structures (A) Electron microscopy and (B) a molecular schema of desmosome. Desmosome are structures responsible for cell-cell contact and also act as keratin nucleation centre. KIF = keratin intermediate filament; PG = plakoglobin; Pkp = plakophilin. (Kitajima, 2012)

The mechanical properties of IFs in the cell depends on their stretchability and elasticity. Those properties are also useful in tissue cohesion.

In tissue, IFs, especially Keratins, act on the cohesion of cells mainly through desmosomes and hemidesmosomes. Desmosomes are the region where the membrane of a cell interlock with the adjacent cell to form tissue integrity (Figure 23). The presence of IFs at desmosomes is well documented (Hatzfeld et al., 2017; Kitajima, 2013). A recent study suggests that keratin is recruited after clustering of desmosome proteins and then elongated at the cluster with desmosome acting as a keratin organisation centre (Moch et al., 2020). AFM based study has shown an increased thickness of keratin filament and differential orientation of the keratin network by a stiffening of the hair follicle by 360-fold along the first millimetre of the follicle. The continued stiffening involved an increased orientation of the network, compaction and mechanical reinforcement of the filament by disulfide cross-link. This study engages the mechanical properties change of the keratin microfibril to its involvement at a tissue scale (Bornschlögl et al., 2016; Hatzfeld et al., 2017). In muscle, Desmin is the main IFs present. Desmin mutations have been linked to muscle weakness disease known as desminopathies. A specific mutation in the Desmin gene called E413K leads to aggregation of Desmin protein, rendering and lowering stress fibre count and therefore reducing traction forces. This leads to the failure of sarcomere proper assembly and lowering muscle contraction (Charrier et al., 2016). This particular mutation does not affect the rigidity of the cell (Charrier et al., 2018). However, Even *et al* (2017) showed using atomic force microscopy that a variant p.D399Y causing aggregation of desmin leads to the increase of cell rigidity, especially at the location of the desmin aggregate (Even et al., 2017). Desmin mutation acting on the cellular level have an impact on the whole muscle function.

II-3-2. Structural role of IFs

II-3-2-1. *At the tissue level*

IFs also have a structural role at the tissue level, controlling shape and tissue organisation. For example, a desmin KO phenotype has a serious effect on skeletal smooth and cardiac muscles formation. Desmin^{-/-} mice heart appears misshapen with extensive areas of fibrosis and calcification with disrupted myocardium fibres. In cardiac muscle as well as skeletal muscles (tongue) disorganisation of the fibres is observed. In concomitance, the stomach smooth muscle layer displays loose tissue organisation (Milner et al., 1996). Thus, desmin has a structural role in the shape and normal muscle formation. IFs play as well a structural role in bone formation, especially IF synemin. Even if the generation of synemin null mice happen to have a normal observed skeleton and formation except for a slight bodyweight decrease, the actual composition of the bone is disrupted. In the long bone, a significant loss of trabecular microarchitecture is observed and a change in cortical bone geometry (Moorer et al., 2016). Trabecular bones exhibit lower calcium content and increase of water absorption making them

more porous (Ott, 2018). The structure of the bones is, therefore, more fragile in the absence of Synemin. The production of serum markers for bone formation (P1NP) is decreased, going with an increase in bone resorption marker (CTX) and therefore the bone mass is reduced (Moorer et al., 2016). Therefore, IFs are important players at the tissue level but also at the cellular level as they maintain cell integrity and structure.

As seen previously thanks to the hemidesmosome containing keratins, they allow cohesion of the tissue allowing the maintenance of the skin integrity. Mutation and defect of keratin leads to skin disorder.

II-3-2-2. At the cell level

IFs being in abundance in certain cell types, they have interactions with different cellular elements and participate in the compartmentalisation of the cell. IFs could be qualified as organisers of the cellular space. They are involved in the positioning of the different organelles, positioning allowing metabolic pathways to happen smoothly. This is the case with autophagy. Vimentin IF seems to be responsible for the positioning and the number of autophagosome and lysosome vesicles in the cell, especially the AA C328 (Pérez-Sala et al., 2015). Inhibition of Vimentin with natural compound Withaferin A causes the aggregation of Vimentin within the perinuclear space. The use of this compound leads to an accumulation of autophagosomes and a clustering of lysosomes in the perinuclear space. Inhibition of Vimentin was found to block the maturation of autophagosome into autolysosome. Using GFP-RFP-LC3 (autophagosome marker) expressed differentially upon the acidity of the lysosome, the fusion of neutral autophagosome and acidic lysosome could be monitored (Biskou et al., 2019). Thus, IFs create an imbalance in the autophagosome distribution as is the case for pigment granules in the skin. Indeed, the IFs network also plays a role in the distribution of melanosomes either in aggregation or isolated forming honeycomb-like structure surrounding the melanosome. An increase anterograde movement of pigment granule is observed in IFs disrupted *Xenopus laevis* melanophores, and a looser network of Vimentin around the granules upon their dispersion revealing a dynamic interaction between IFs and melanosome and a possible modulator role of IF in their movement (Chang et al., 2009). IFs are surrounding mitochondria in different ways, either by confinement or direct binding via the plectin protein and become looser during signalling (Figure 24) (Schwarz & Leube, 2016).

Therefore, IFs having a tight connection with mitochondria are involved in their distribution and motility. The motility rate of mitochondria is increased in cells with a disrupted Vimentin network. This phenotype can be rescued by re-expressing wild-type Vimentin (Nekrasova et al., 2011). Aberrant subcellular distribution is observed in Desmin knockout (Winter et al., 2016).

In primary murine myotubes transduced by lentiviral containing Desmin rod and tail domain mutation display an underdeveloped mitochondrial network featuring mitochondria with less filamentous structure. The differential shape and distribution in comparison to the wild type contribute to an impaired function of the mitochondrion, with the respiratory chain being impacted (Smolina et al., 2020). IFs interaction with mitochondria is similar to their interaction with the nucleus in the sense that the same crosslink protein is needed: plectin.

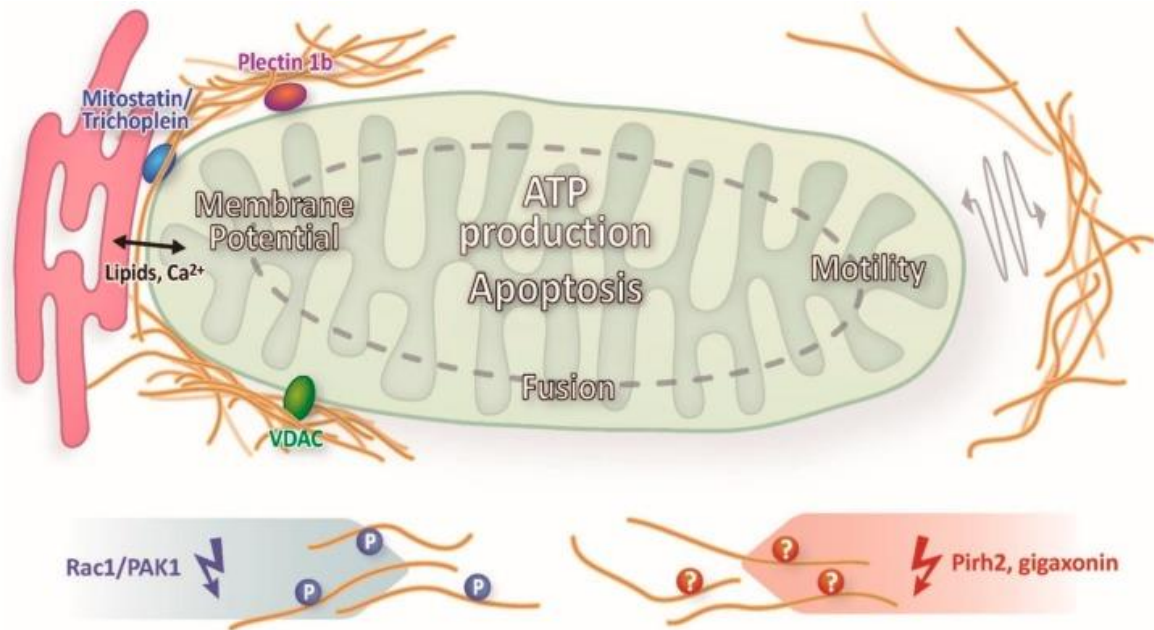


Figure 24 Interaction of vimentin with mitochondria. Vimentin is involved in three ways with the mitochondria. Firstly, by direct contact with the organelle outer membrane via the protein plectin 1b and VDAC, secondly, by confining it by forming a cage around it, controlling its movement within the cell and by relaying the signal coming from it such as Rac1/PAK1 and Pirh2, gigaxonin pathways (Schwarz & Leube, 2016).

This anchorage of the nucleus allows IFs to be involved in its positioning. Forming a cage-like structure around it, IFs actively participate in the nuclear movement in the cell, especially during polarisation in primary rat astrocytes. When IFs are depleted, nuclei lose their back position on crossbow micropattern and become more centred. During the migration of the cell, the normal rotation of the nucleus is not observed (Dupin et al., 2011). For more details on the nucleus positioning, see IV-3. It has been recently observed that Vimentin levels are involved in nuclear shape regulation as well as chromatin condensation in a study using mesenchymal stem cells (Keeling et al., 2017).

II-3-3. IFs involvement in signalling

IFs not only serve as a structural scaffold but also form a molecular scaffold that connects with signalling pathways to influence cell behaviour in physiological and pathological situations (Pallari & Eriksson, 2006). The connection of IFs with intracellular structures parallels the ability of these filaments to influence cellular functions. While focal-adhesion-mediated signalling influences the organization of the IF network, IFs can in turn influence the dynamics of FAs. They participate in focal-adhesion-associated signalling, as shown for vimentin, which regulates the expression level and the localization of FA kinase (FAK) and the Rac1 guanine nucleotide exchange factor Vav2 (Havel et al., 2015). In addition to their role in controlling actomyosin contractility, vimentin and more generally type III IFs facilitate cell migration and invasion of mesenchymal cells by controlling the dynamics and distribution of FAs (De Pascalis et al., 2018; Mendez et al., 2010; Menko et al., 2014). Depending on the cell type, IFs have different effects on cell migration that may be explained by the difference in IF proteins or integrin expression patterns. Mirroring their protective role against mechanical stresses, the signalling functions of IFs are also involved in cellular survival by promoting cell-cycle progression, maintaining organelle homeostasis and protecting against apoptosis. Most IF proteins, including keratins, GFAP, vimentin and neurofilament proteins, interact with 14-3-3 proteins.

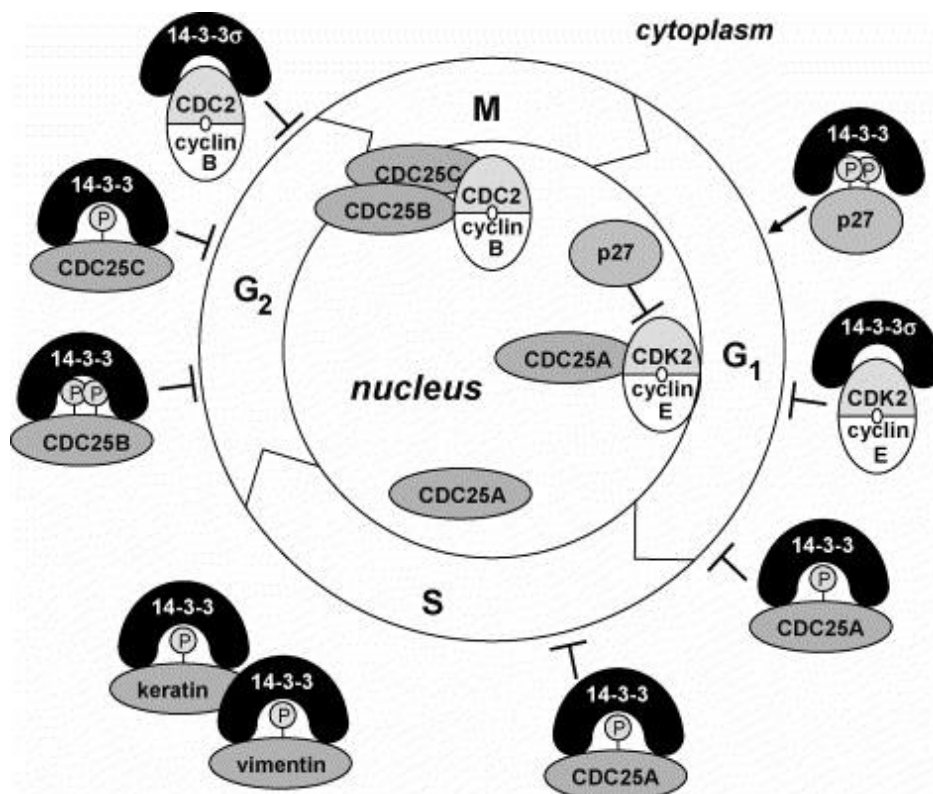


Figure 25 Keratin role during mitosis. Phosphorylated keratin and vimentin are sequestered during cell cycle phase S by the protein chaperone 14-3-3 ↑, Activation of cell cycle progression; ↓, inhibition of cell cycle progression (Hermeking & Benzinger, 2006)

The 14-3-3 family of proteins forms a major class of molecular chaperones, binding several kinases including Raf, protein kinase C (PKC), c-Bcr, Bcr–Abl and phosphatidylinositol 3-kinase, thereby regulating their activity, localization or stability (Mhawech, 2005). By controlling 14-3-3 localization, IFs are well-positioned to influence a wide range of vital regulatory processes, such as mitogenic signal transduction, apoptotic cell death, and cell-cycle control (Figure 25) (Hermeking & Benzinger, 2006). 14-3-3 interactions with target proteins may participate in the formation of protein aggregates, such as those observed in neurodegenerative diseases, and may regulate pathogenic processes (Shimada et al., 2013). Other signalling functions have been described for keratin IFs. Keratin 8–18 IFs interact with the cytoplasmic tail of tumour necrosis factor (TNF) receptors to influence their signalling and protect cells from apoptosis (Caulin et al., 2000). The same keratin 8–18 IFs have also been shown to interact with and activate Notch1 and promote epithelial differentiation in the large intestine (Lähdeniemi et al., 2017). In the case of Notch regulation, vimentin can also play a crucial regulatory role: in endothelial cells, vimentin regulates Notch signalling strength and arterial remodelling in response to hemodynamic forces, suggesting that several IF proteins may contribute to the regulation of the Notch pathway (van Engeland et al., 2019). In the nucleus, lamins control nucleocytoplasmic transport and gene expression: increased lamin B1 expression in huntingtin-mediated neurodegeneration affects the chromatin domains associated with lamins, chromatin accessibility and transcriptional regulation (Alcalá-Vida et al., 2021). We are probably only scratching the surface when considering IF involvement in biochemical signalling. How the composition of IFs can modulate intracellular signalling is also a broad question that systematic proteomic analysis of IF molecular partners may help to answer. In addition, recent evidence reveals a role for soluble IF proteins in IF signalling functions (Ikegami et al., 2020; Torvaldson et al., 2015). Phosphorylated soluble lamins interact with gene enhancer regions to control gene expression, suggesting that local or partial depolymerization of IFs may provide soluble IF proteins that serve as signalling intermediates (Dutour-Provenzano & Etienne-Manneville, 2021).

II-4. Key messages

- A. The cytoskeleton components are the guardian of the shape and organisation of the cell and at a larger scale, the tissues. To do so, the three components have complementary physical and mechanical properties. Actin is mainly responsible for the shape of the cell and its contractility, thanks to the actomyosin network. MTs are more flexible than actin.
- B. Intermediate filaments have very interesting mechanical properties. Their elasticity is incomparable, they are highly resistant to breakage by stretching. They are surrounding organelles within the cytoplasm and partake in vesicular transport. These mechanical properties and localisation make them great organisers of the cellular space. They are also involved in tissue integrity and cohesion through desmosomes and FAs.
- ➔ IFs have mechanical properties that point to a role in cellular mechanics. However, their exact role in cell mechanics still remain to be elucidate. Could they be implicated in the cortical, cytoplasmic or nuclear rigidity? Could they transmit forces if they cannot generate them? IFs, actin and MTs are interacting together for a lot of different cellular function, therefore we could ask ourselves how IFs integrate with other cytoskeletal network in mechanical pathways?

Mechanotransduction

III. Mechanotransduction

III-1. Mechanotransduction definition

Cells continuously adapt to their microenvironment. In particular, they modulate their morphology, growth, division, and motility depending on the biochemical and the physical properties of the extracellular matrix (ECM) (Timraz *et al.*, 2015). Mechanotransduction is the capacity of the cell to sense mechanical signal from its surrounding, as in cell-cell contact or, here more described, the ECM, and convert it into biochemical activity and downstream signalling (Figure 26). Several cell structures are involved in this process usually starting at adhesive structures. Here we exemplify FAs. These structures allow cells to interact with ECM proteins through the core transmembrane proteins called integrins and to sense the nature and rigidity of the ECM proteins (Seetharaman & Etienne-Manneville, 2018). The mechanical signal is transduced by FAs proteins and often translated into changes in actomyosin-mediated mechanical tension (Sharkar *et al.*, 2020; De Pascalis *et al.*, 2018). Downstream signalling pathways can also reach the nucleus. Gene expression can be then modified which may in return affect the composition of FAs for an adaptative cell response (Figure 26) (Jonata *et al.*, 2020). This process allows the cells to respond and adapt to the changes in the physical and mechanical properties of the ECM.

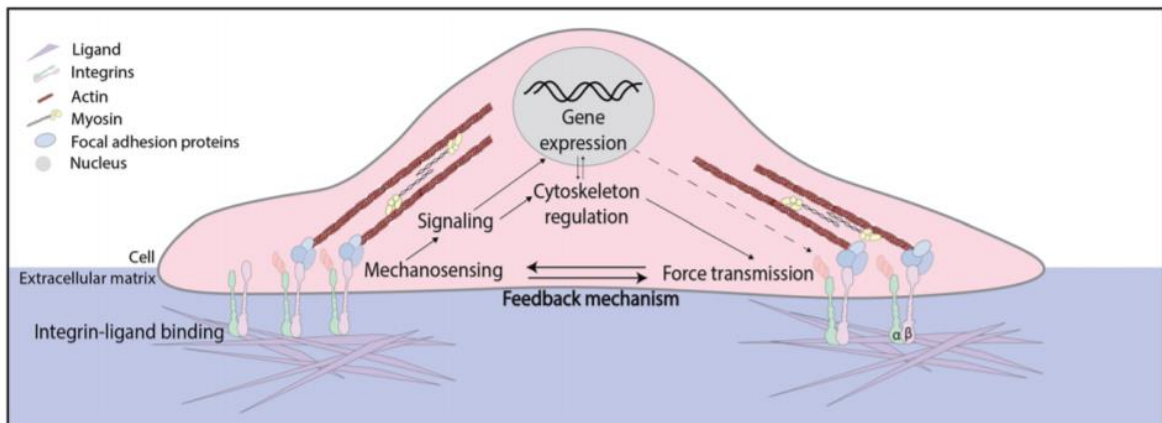


Figure 26 Cells attach to the ECM via integrin-mediated adhesions that help the cell sense the ECM mechanical properties and convert them into biochemical signals. These biochemical signals initiate a cascade of signalling pathways to affect the cytoskeletal organisation, gene expression and eventually, cellular functions. This entire process by which a cell responds to the mechanical cues from the surrounding environment is termed mechanotransduction (image and caption by Seetharaman and Etienne-Manneville 2018).

Mechanical signals from the ECM to the cell are of different nature. They are mainly linked to the density of ligand present in the ECM, its porosity, elasticity, its topography and the rigidity of the substrate. The effect of the density of the ligand on mechanotransduction is a developing field and starting to be recognised in cancer research and new technology are now available to

study its effect as developed in more detail in this review (Amschler & Schön, 2020). Ligand density such as collagen surface density is affecting cell spreading, and a medium-range and equilibrium of density of collagen are needed for maximum spreading. Passing a density point, the cell area decreases (Gaudet et al., 2003). It also affects the cytoskeletal organisation and tension on the nucleus (Stanton et al., 2019).

The topography at a nanoscale represents the structures on which the cells are in contact. Topography influence cell fate, cell adhesion, cytoskeletal organisation, apoptosis, contact guidance, gene expression (Britland et al., 1996; Clark et al., 1987, 1991; Dalby et al., 2004). The structure of the microenvironment triggers the mechanotransduction pathway that will determine cell fate. *In vitro*, scientists came up with several ways to change the topography such as microgrooves, nanopits, nanocolumn and pillars, nanoislands set from many examples, as described in figure 27. These technologies are especially used in stem cell and cell engineering research where mechanotransduction pathways are used to differentiate cells depending on the topography.

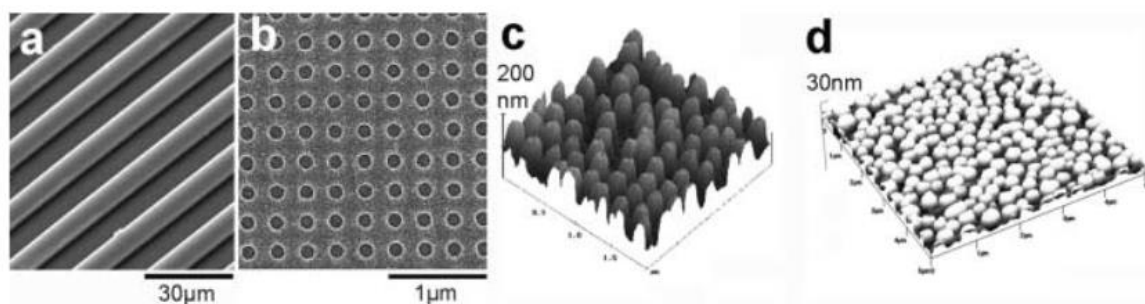


Figure 27 Microscopic images of different topography. (a) Micro-grooves (10µm period, 200 nm depth) produced by photolithography and dry etch (image courtesy of Mrs M. Robertson). (b) Nanopits (120 nm diameter) produced by electron beam lithography and dry etch (image courtesy of Dr. N. Gadegaard). (c) Nanocolumns (160 nm tall, 100 nm diameter) produced by colloidal lithography. (d) 27 nm high polymer demixed (polystyrene/polybromostyrene) nanoislands (courtesy of Dr. S. Affrossman). Image and caption from (Dalby et al., 2004).

Nano-scale vibration composed also part of the mechanical signals received by the cells. It is particularly important in stem cell differentiation into osteoblast. Bone cells are exposed to vibration *in vivo* through shock via movement and gravity. A method developed by the lab of Prof. Dalby, called nanokicking allows stem cell to differentiate into osteoblast by only applying nanovibration, without the supplementation of specific growth factor. This nanovibration induce cytoskeletal reorganisation and the activation of mechanical pathways (Campsie et al., 2019; Hodgkinson et al., 2021). More details of this technique can be found in this review (Robertson et al., 2018).

In this study, however, we focused more on mechanotransduction through rigidity sensing, which is described in the following part.

III-2. Substrate Rigidity

The rigidity of the surrounding microenvironment can determine the cell fate, morphology and provoke changes in cellular processes. Rigidity sensing is crucial in health, as the human body possesses different rigidities, the bone being the stiffest, and the brain having the softest tissues (Figure 27). Within tissues, however, some structures can display different rigidity. In brain tissues, different rigidities can be observed: where most of the tissue is soft, blood vessel linings and neuron myelinated axons have higher rigidities. The stiffness of the tissue influences the differentiation, the lineage of the cell, i.e., mesenchymal stem cell in a rigid environment will take bone cells features and inversely, a softer environment leads to neuron-like features (Engler et al., 2006). Rigidity sensing affects cellular migration and is of particular importance in malignancy (Paszek et al., 2005). The tumour microenvironment frequently undergoes transitions to higher stiffness.

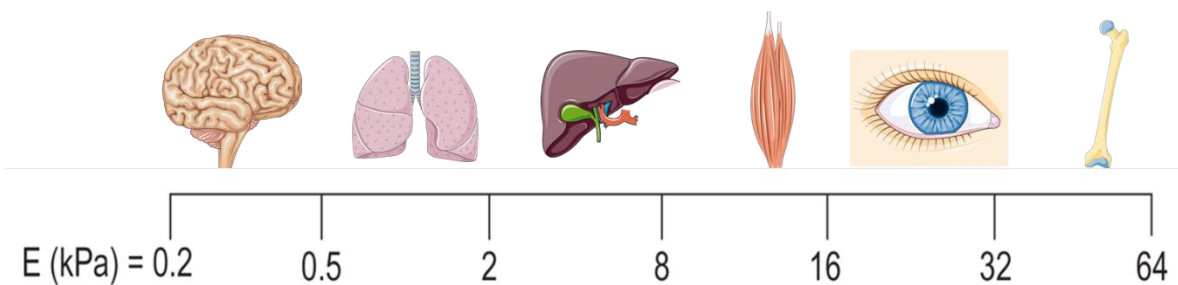


Figure 28 Rigidity of the substrate in different human tissue. This scheme represents a range of rigidity across the human body, starting by the softest tissue: the brain, ending with the stiffest structure: the bones. The brain stiffness however naturally ranges between 0.2 and 2 kPa

Cells are testing the rigidity of the ECM by applying forces through early adhesion complexes and by generating a constant shear force on the ECM that allows scaling roughly the area before maturation and engagement of FA (Ghassemi et al., 2012).

Cells behave and are shaped differently depending on substrate rigidity. For instance, on a softer substrate, they tend to exert fewer traction forces than on a stiff substrate. This correlates with FAs appearing smaller and more dynamic on soft surfaces, and bigger and more stable on stiff surfaces (Ghibaudo et al., 2008; Seetharaman et al., 2020). However, sensing and adapting to the different rigidity is not only a question of FA morphology. FAs are recruiting proteins and effectors, and several post-translational modifications are necessary for FA engagement in the ECM (Trichet et al., 2012). The cell is constantly testing the rigidity; hence a high turnover rate is needed.

Note:

Mechanotransduction is the mechanism by which the cell sense and respond to changes in the mechanical properties of ECM such as changes in substrate rigidity. Brain is the softest tissue, with a range of 0.2 to 2kPa Young modulus.

III-3. Mechanosensing at adhesive structures on different substrate rigidity

III-3-1. Mechanosensing at FAs

FAs are crucial structures that allow the cells to attach to the ECM, but also are crucial mechanosensory signalling nodes that sense the extracellular chemical and mechanical signals. FAs play the role of bidirectional interfaces between the outer microenvironment and the inner cell. FA are linking the ECM and the inner cell cytoskeleton (Figure 28). They are very complex structures comprising more than 150 proteins that self assemble and elongate following tension and disassemble when the tension is lower (Geiger & Yamada, 2011; Shemesh et al., 2005). Several of them are key actors in the process of rigidity sensing and adaptation to the ECM.

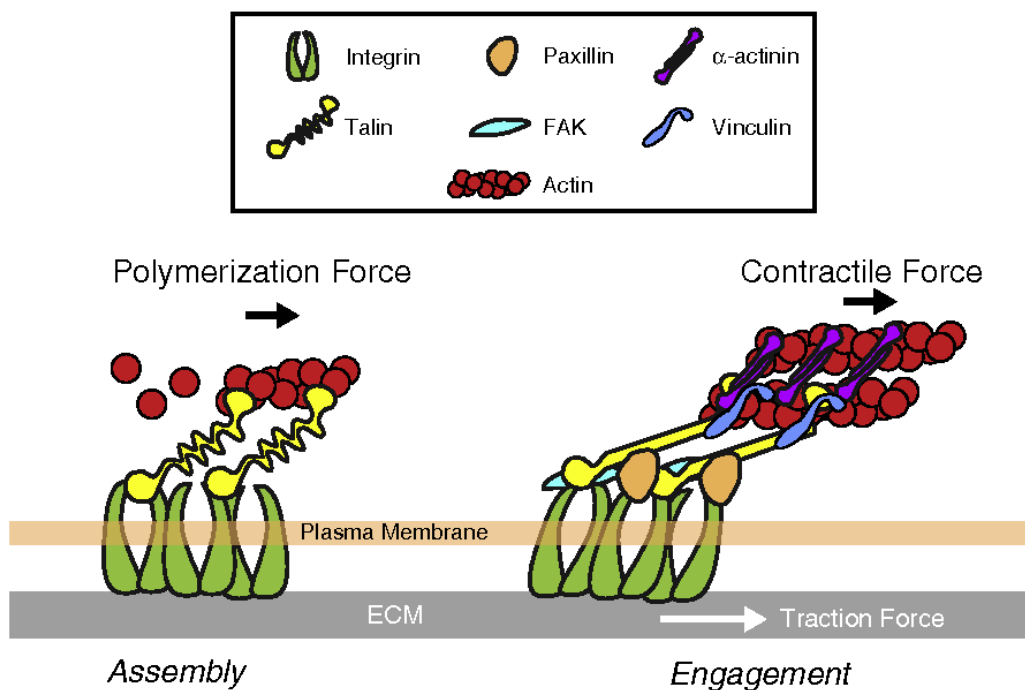


Figure 29 FA and actin polymerisation and contractile forces. Force transmission by FAs during nascent adhesion assembly (left) and after engagement to the ECM and production of traction force (right). Contractile forces following engagement of integrin on the ECM lead to the tension of talin spring-like protein. (adapted from Oakes and Gardel, 2014)

Indeed, some are recruited and stay for the entire lifetime of the FA, like integrin and talin, and others are recruited only when tension becomes higher, like vinculin (Oakes & Gardel, 2014; Shams, 2016). The transmembrane part of the complex is assured by proteins called integrins. Integrins are clustered together through directed actin polymerization at the lamellipodia

(Galbraith et al., 2007). Integrins are found in two different states: in a bent conformation in an inactive state with the cytoplasmic tails close together, and straighten when engaged, with cytoplasmic tails apart (Seetharaman & Etienne-Manneville, 2018). Integrins are linked to the protein Talin that acts as a mechanically activated spring (Figure 28). When integrins are engaged and anchored to stiff substrates, talin changes conformation through a process called mechanosensing and triggers the recruitment of FA proteins such as paxillin and vinculin that are linking the actomyosin structures called stress fibres (Elosegui-Artola, 2017). The activity of the FA is associated with the phosphorylation status of its component provided by FA Kinase (FAK). Activation of FAK leads to the formation of a complex with Src proteins responsible for the activation of downstream signalling pathways through the phosphorylation of several FA effectors such as p130cas (X. Zhao & Guan, 2011). There is an extremely rapid turnover of proteins at FAs, with some FA proteins such as talin, FAK, zyxin, α -actinin or vinculin life time being less than 30s, according to fluorescence recovery after photobleaching (FRAP) experiments (Shams, 2016; von Wichert et al., 2003).

III-3-2. Mechanosensing at adherens junctions

Adherens junctions are complexes linking cells together. These cell-cell junctions are crucial for the maintenance of tissue cohesion. Tissues can be put under stress and need to exert forces to withstand breakage. This is where adherens junctions play an important role. The main adhesive protein present in these structures are cadherins. In adult tissues, they are extremely important in the rapid growth of tissue such as the gut lining and brain plasticity with the regulation of neuronal synapses adhesion (Gumbiner, 2005). They are therefore indispensable for the shaping of multicellular bodies and also the transmission of mechanical loads between cells (Ladoux et al., 2015). An imbalance in forces at cell-cell junction leads to cell contact and shape remodelling, progression or migration (Montell, 2008; Takeichi, 2014). AJ's are linking directly transversal arcs of actin through the protein α -catenin, which in turn recruits vinculin for a complex with the actomyosin contractile cables. In this study, we looked at sparse cells that do not form adherens junctions, thus, we focused here further on FAs.

III-4. Cytoskeletal network in mechanotransduction

Cells attaching to the substrate and sensing the rigidity is triggering signalling cascades that in turn are affecting cytoskeleton dynamics and organisation. Mechanotransduction goes with the regulation of traction forces at adhesive structures for an adequate adaptation to the ECM properties. Thus, mechanotransduction at FA leads to effective changes in the cytoskeleton network. In response to the sensing of the ECM, cytoskeleton proteins are reorganising, forming new or different structures.

III-4-1. Actin

III-4-1-1. Role of actin/actomyosin in mechanosensing

Actin is polymerising to develop stress fibres for force application on the substrate. The cytoskeleton especially actin is regulated by a small GTPase, particularly from the RhoA pathway. Mechanical tension at the plasma membrane activates the RhoA pathway through RhoGEF, located at the plasma membrane that activates RhoA through endogenous G-Protein Coupled Receptors for a RhoA dependent actin polymerisation (Figure 29) and makes the binding sites of mechanosensor accessible (Hoon et al., 2016; van Unen et al., 2015).

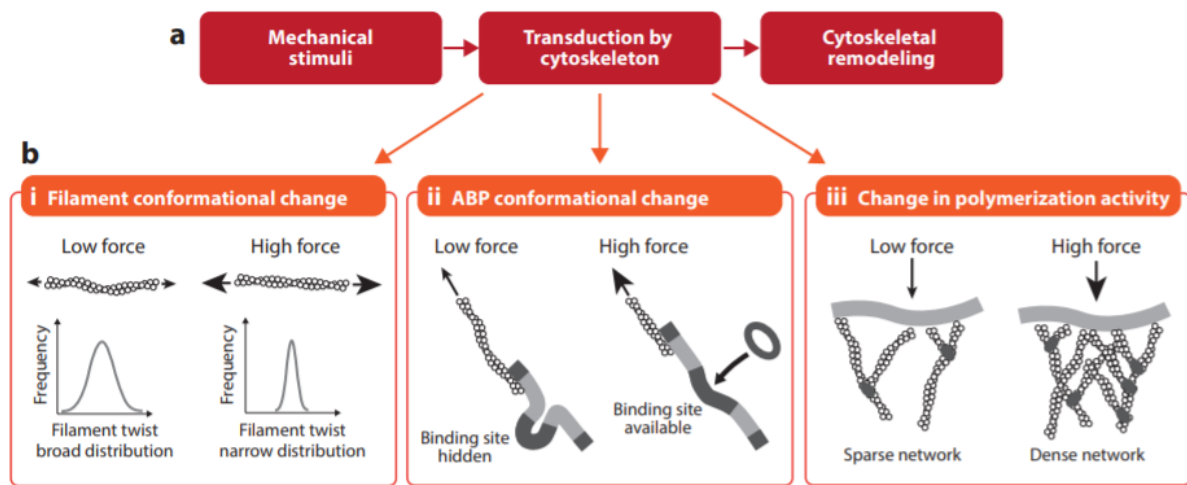


Figure 30 Mechanical force transduction by the actin cytoskeleton. (a) Mechanical forces on cells are transduced by the actin cytoskeleton into biochemical signals. These often culminate in cytoskeletal remodelling as the cell responds by changing shape. (b) We highlight three main modes for mechanical force transduction by the actin cytoskeleton. (b, i) Firstly, actin filaments themselves can be susceptible to mechanical forces, changing conformational state under mechanical load. (b, ii) Secondly, actin-binding proteins can change conformation under mechanical load, exposing binding sites for other proteins that were previously unavailable. (b, iii) Finally, the polymerization kinetics of actin-binding proteins can be influenced by the mechanical load upon them, thus changing the network density and growth rate. Abbreviation: ABP, actin-binding protein. (Caption and image by Harris et al., 2018)

Activated RhoA boost the polymerisation of actin via the Rho GTPase effector formin. Formin interacts with straight actin and polymerises its barbed end (Kühn & Geyer, 2014). Upon mechanical tension, actin polymerisation is also increased by the inactivation of cofilin, an actin severing activity protein, through the phosphorylation of LIMK1 by the RhoA effector ROCK. ROCK is simultaneously phosphorylating myosin light chain allowing the increase of cellular tension through the actomyosin network (Hoon et al., 2016).

III-4-1-2. Impact of mechanotransduction on actin and forces.

Actin is thus the main cytoskeletal actor when it comes to the generation of traction forces. Actin is also in direct contact with the mechanosensing machinery at FAs (Harris et al., 2018).

After mechanical stimuli, the actin network is completely reorganised (Figure 31). To relay back to substrate rigidity, on stiffer substrates, actin is forming stronger stress fibres, resulting in higher forces exerted on the substrate, as depicted in figure 30b iii and figure 30. The effect of substrate rigidity is mediated by the regulation of actomyosin through Rho and myosin light chain phosphorylation. RhoA activity increases with stiffness, leading to increased myosin light chain phosphorylation that activates the movement of the myosin head and sliding of the myosin along actin filament resulting in higher contraction in stiffer substrates (Harris et al., 2018). The role of actomyosin in generating forces has been extensively studied, fewer is known about the role of MT and IFs in mechanotransduction.

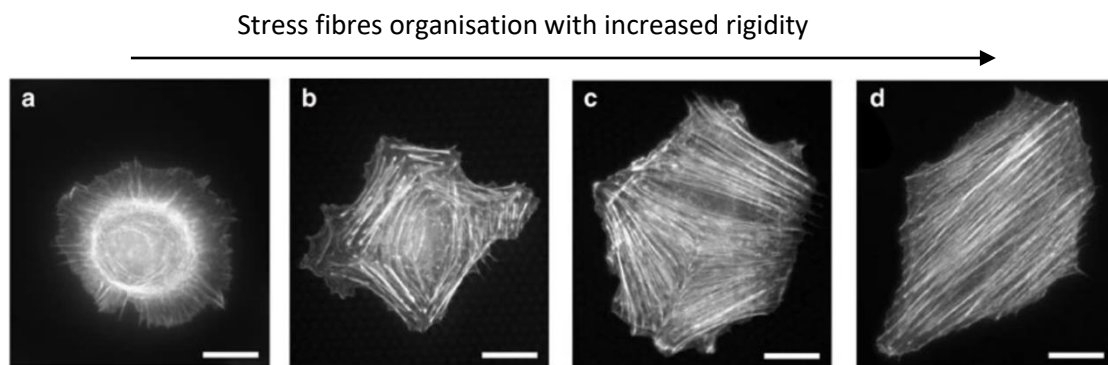


Figure 31 Actin organisation in substrate rigidity increase. REF-52 cells on micropillar substrate of increasing rigidities -9nN/ μ m, 43nN/ μ m, 64nN/ μ m and 85nN/ μ m. The cells were stained for F-actin, scale bar: 20 μ m (adapted from Gupta et al., 2015)

III-4-2. Microtubules

MT roles in mechanotransduction are more elusive and less studied than the role of actin. MT are a good candidate to study mechanotransduction due to their incredible stiffness, three folds higher in magnitude than actin (Gittes et al., 1993) and are stabilised under tension as seen in *in vitro* studies (Hamant et al., 2019; Kabir et al., 2014). Recent studies from our lab showed that MT acetylation affects FAs, studied via the depletion of the alpha Tubulin Acetyl Transferase 1 (α TAT1). Depletion of this enzyme altered the number and the localisation of FAs in migrating astrocytes. They showed that acetylated MTs and α TAT1 localise close to FAs. There has been no evidence as to date that MT interact with FAs directly, but they come in very close proximity, and MT binding proteins are enriched at integrin based complexes containing FAs proteins (Byron et al., 2015; Seetharaman & Etienne-Manneville, 2019). In recent years, MTs have been put in front of the scene as cell adhesion turnover regulators, piling on to pioneer studies about MT and FAs interaction and control. Nocodazole drug depolymerisation of MTs leads to increase number and size of FAs and regulation of small GTPase Rho that in turn induce the formation of large FAs and higher contractility (Bershadsky et al., 1996). MTs are guided towards FAs along the actin cables. MTs in the vicinity of FAs give them a potentially important

role in mechanical responses. Further studies from the lab showed the importance of MT and particularly MT acetylation in tuning mechanosensitive responses. They showed that actomyosin dependent sensing of the substrate rigidity controls the acetylation of MTs via the recruitment of α TAT1 to FAs. In turn, the acetylation of MT leads to the activation of RhoA through GEFH1 which promotes actomyosin contractility and traction forces on the substrate (Seetharaman et al., 2020). This highlights the importance of MT stabilisation in mechanotransduction at FAs.

III-4-3. Intermediate filaments

The role of IFs in mechanotransduction has not been clearly defined and a lot is to unveil about their interactions with FAs. However, certain IFs could potentially interact directly with FA by binding to integrin $\alpha 2\beta 1$ such as synemin, but most binds indirectly via the protein linker

Focal adhesions

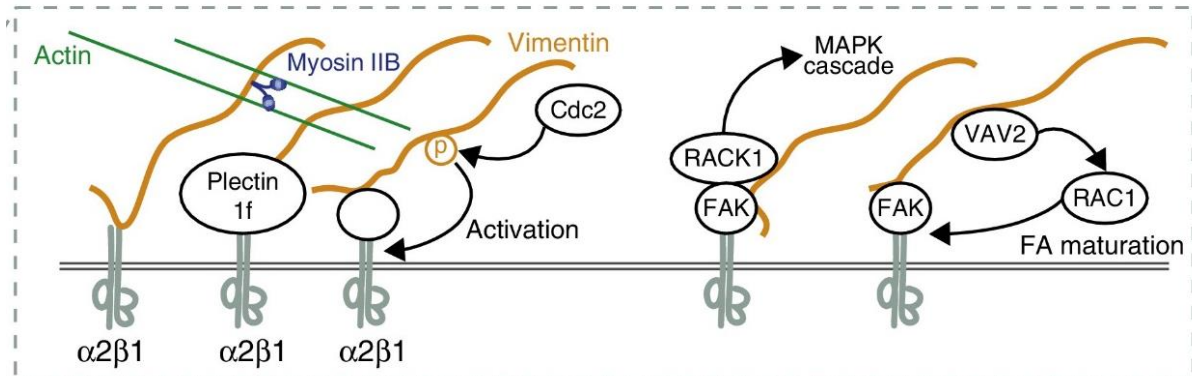


Figure 32 Vimentin interaction at FA. Vimentin can interact with FAs through different mechanisms: directly by interacting with integrins or via FAs protein such as FAK or Plectin1f (adapted from Leduc & Etienne-Manneville, 2015).

plectin1f (Figure 29). This is well observed in the case of vimentin. When vimentin is decoupled from FAs either by vimentin deficiency or the inhibition of the protein linker plectin, there is a decreased activation of the mechanosensory protein FAK (FA Kinase) and its downstream effectors. There is therefore a strong decrease of FA turnover rates in fibroblast, associated with an upregulation of the feedback loop acting on RhoA and myosin light chain (Gregor et al., 2014). It has also been described that a lack of IFs leads to the lower turnover rates of FA characterised by longer structures in a migrating glial cell model (De Pascalis et al., 2018). Affecting turnover and structural morphology of FA might lead to the impairment of mechanosensing of the ECM and in turn affect cellular pathways such as migration. A lot of suspicions give a role of IFs in mechanosensing at FAs, due to their interaction, their specific mechanical properties and their regulatory effect on the other cytoskeleton components. A lot still needs to be deciphered.

Upon mechanical stress, IFs mechanical properties come in handy. Their elastic properties and their abundance as well as their involvement in several signalling pathways in the cell, make them great potential actors in the mechanical response. A study in the lab started to depict more closely the role of IFs in mechanotransduction during collective cell migration and showed that IFs regulated traction forces (De Pascalis et al., 2018). In this study, it was shown that IFs control the distribution and the strength of traction forces across a migrating monolayer. Migrating cell size was increased after depletion of IFs using siRNA against Vimentin GFAP and Nestin, with slower migration. Traction forces microscopy experiment showed that depletion of IFs generates more traction forces and throughout the monolayer (followers and leader cells) than control cells where the forces are present more at the leader cells. This suggests that IFs are restricting the generation of traction forces to the leader cells and preventing the accumulation of forces throughout the migrating monolayer allowing faster migration. IFs also control the organisation of the actomyosin network. In triple IFs, actin structures are more perpendicular to the wound with lower actin and N-cadherin retrograde flow, impairing cell-cell junctions. The paper also suggests that IFs exert control over FAs dynamic and turn over by interacting with them through plectin. In triple depleted IFs migrating astrocytes, FAs are more dispersed within the cell. The lifetime of FAs is greatly increased in this condition, leading to a slower turnover, most likely linked to the high traction forces exerted on the substrate. In addition, the mechanosensory protein vinculin, usually present at FAs and AJs, is absent from AJs. Using vinculin fluorescence resonance transfer (FRET) tension sensor, the tension exerted on vinculin was quantified throughout the migrating monolayer. FRET index was lower on IFs depleted cells, meaning that vinculin-mediated tension was increased in those cells. The effect of the depletion of IFs on traction forces and distribution of FAs in migrating cells being similar to siRNA knockdown of plectin effect, it is strongly suggested that IFs and plectin act together at FAs to regulate actomyosin network and transmission of forces (De Pascalis et al., 2018).

Signalling cascades downstream of membrane receptors can locally influence the dynamics and properties of IFs; for example, Rho GTPases and their effectors PAK and ROCK modulate IF protein phosphorylation, and regulation of Rho signalling downstream of integrins can alter the local organization of the IF network (Goto et al., 1998). In migrating epithelial cells, the global redistribution of polymerization sites towards the leading edge facilitates the generation of new filaments and organizes the turnover of the network (Kölsch et al., 2009). In contrast, in migrating astrocytes the polarization of the IF network relies on increased kinesin-mediated transport of vimentin and GFAP-containing IFs towards the leading edge, coupled to a local integrin-dependent Cdc42-driven inhibition of their dynein-mediated retrograde transport (Leduc & Etienne-Manneville, 2017). Focal-adhesion-mediated signalling also regulates

vimentin phosphorylation. Fibroblasts plated on soft substrates show increased vimentin phosphorylation associated with an increase in the soluble pool of vimentin, suggesting an adaptation of the properties of the network to the mechanical characteristics of the cell microenvironment (Murray et al., 2014). Cell stretching has been recently shown to increase the tension of keratin fibres, thereby controlling their interaction with C-terminal tensin-like protein (CTEN/tensin 4), to influence FA signalling (Cheah et al., 2019; Dutour-Provenzano & Etienne-Manneville, 2021).

Whether substrate rigidity and mechanosensing at FAs can influence IF organisation is still an open question that I tackled during my PhD. To understand how IFs contributes to mechanotransduction I also investigated their possible impact on the nucleus.

III-5. Key messages

- A. Mechanotransduction is an extremely important process for the cell adaptation to its environment. The cell picks up on external mechanical cues and transforms them into biochemical signals. The cells sense, through adhesive structures such as FAs, the changes in ECM properties.
 - B. Cells have to adapt to the rigidity of their microenvironment through the appropriate organisation of the cytoskeleton.
 - C. Very little is known about IFs involvement in mechanosensing and the overall mechanotransduction machinery. We previously described the regulation by IFs of the traction forces of the actomyosin network during collective cell migration.
- ➔ Although our previous study and others showed that IFs can regulate actomyosin contractility, two main questions remain unsolved: Do IFs directly or indirectly participate in mechanosensing? How do IF control Rho-dependent contractility?

Mechanotransduction at the nucleus

IV. Mechanotransduction at the nucleus

Mechanotransduction focused on how mechanical forces affect cell and tissue behaviour (Jansen et al., 2015). In the last few years, emerging evidence put the nucleus at the centre of mechanotransduction. Astonishingly, recent data found that the nucleus is used as a ruler to measure the degree of spatial constrain. In confinement, the deformation of the nuclear envelope and its elongation activate signalling pathways to the actomyosin network that tailor the contractile response to the confinement degree (Lomakin et al., 2020). The active role of the nucleus in mechanical pathways stand from his size and stiffness. The nucleus is the stiffer and larger organelle in the cell. Its stiffness is attributed to two important components: the meshwork of lamin IFs and the chromatin structure. Lamins are interacting with the cytoplasmic cytoskeleton and act as a shock absorber to prevent too serious deformation of the nucleus under tension (Dahl et al., 2004; C. Y. Ho et al., 2013). Knowing more about the link between the nucleus and cytoplasmic cytoskeleton seems indispensable to get a better understanding of mechanotransduction pathways.

IV-1. The LINC complex connects the cytoskeleton to the nucleoplasm

The nucleus is the biggest organelle of the cell. The cytoskeleton proteins are known to be linked to the nuclear envelope through the LINC (Linker of the Nucleoskeleton and Cytoskeleton) complex, an assembly of protein anchoring the cytoskeleton to the inner meshwork of lamin IF (Starr & Fridolfsson, 2010). The composition of the LINC complex is depicted in figure 33. Each of the cytoskeletal proteins has a specific way to attach to the nucleus via a different component of the LINC. Actin interacts directly via nesprin 1/2, IFs via nesprin 3 through the intermediary of plectin, and MT to nesprin 4 via the kinesin Kif5B (*Figure 33*) (Dupin et al., 2011; Mellad et al., 2011). Nesprin links SUN domain protein in the interstitial space between the outer and inner membrane. The LINC complex is of utmost importance in the transmission of the mechanical signal. To reach the inner nucleus and alter gene expression and exert changes in the nucleus, the mechanical signal is helped by the LINC complex. The nesprins, anchoring the outer membrane of the nucleus and linking the cytoskeleton to it, are therefore able to transmit the tension (Déjardin et al., 2020). Nesprin 1 and 2 as well as SUN 1 are essential for nucleus rotation and reorientation upon cell stretching. Depletion of Nesprin1 leads to a higher number of FAs and increased traction forces on the substrate (Chancellor et al., 2010). These studies point towards the LINC complex as a major player in mechanotransduction, suggesting a feedback mechanism between the nucleus and FAs.

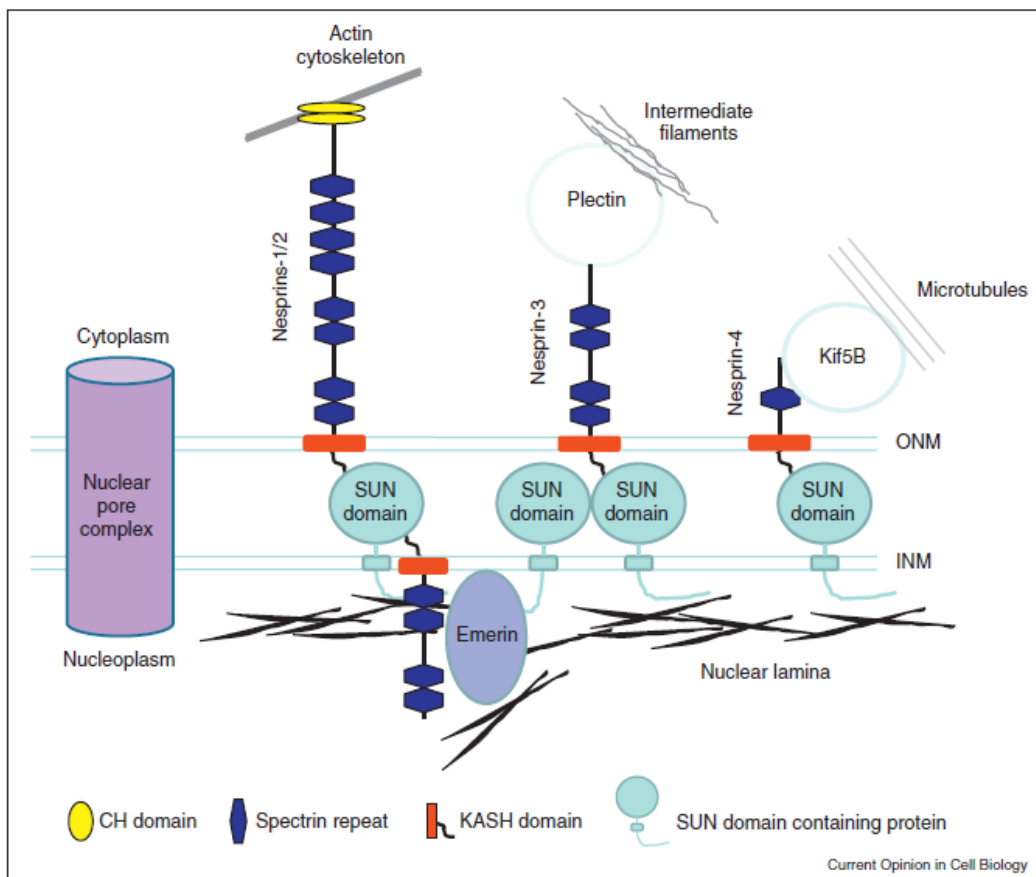


Figure 33 The LINC complex is needed to anchor the cytoskeleton to the nuclear lamina. The LINC complex is composed of SUN domain proteins (SUN1-2, UNC84 and SPAG4). Smaller nesprin-1/2 isoforms as well as emerin binds lamina through interaction of lamin A on the inner membrane. On the outer membrane, the KASH domain of the larger isoforms of nesprin-1/2, nesprin-3 and -4 bind to the SUN domain within the luminal space to anchor nesprin to either actin, MT or IFs. IFs interact with nesprin-3 via the protein linker plectin (Mellad et al., 2011).

IV-2. The cytoplasmic and nuclear cytoskeleton protect the nucleus from mechanical stress

IV-2-1. Lamins

Linking lamins is therefore an important task for signal transmission: ratio of Lamins A/C and B1 are altered by tension and substrate rigidity and leads to differential gene expression (Lammerding et al., 2004; Swift & Discher, 2014). The nuclear IF lamin A/C is involved in the intranuclear organisation in an emerin dependent manner. In DLD-1 colorectal adenocarcinoma cells, lamin A/C impacts the organisation and the dynamic of chromatin in the interphase nucleus. The co-depletion of lamin A/C and emerin via siRNA leads to a missed location of chromosome territory position observed in a Fluorescent In-situ Hybridisation of Chromosome Territory CT18 and 19 (Ranade et al., 2019). The organisation of chromatin is very important for smooth gene expression and the cell put in place systems to protect the nucleus.

The intranuclear meshwork of lamins plays a fundamental role in nuclear shape and mechanics and the elastic capabilities of lamins are crucial in upholding nuclear integrity during cell compression. Lamins also affect chromatin dynamics in the interphase nucleus and may thereby influence nuclear functions. Because of their tight interaction with the nuclear envelope, and their unique mechanical properties, IFs serve to protect nuclear integrity and probably also influence the mechanosensitive organization and functions of the nucleus. In skeletal muscle cells, lamin A mutations related to muscular dystrophy cause transient rupture of the nuclear envelope, resulting in DNA damage, activation of the DNA damage response, and reduced cell viability (Earle et al., 2020). Moreover, like cytoplasmic IFs, lamins are also important in protecting the cell and the nucleus against oxidative stress: a higher percentage of ROS is found in patient fibroblasts expressing mutated lamin A following induction of oxidative stress (Dutour-Provenzano & Etienne-Manneville, 2021).

IV-2-2. Actin cap

As mentioned previously, the LINC complex and its interaction are the first lines of defence of the nucleus against compressive forces. Interestingly, lamin A/C can organise a perinuclear cap of actin to resist nuclear deformation induced by mechanical stress. Researchers found an increase of perinuclear actin in wild type cells with stretching. This actin cap is lost in laminA deficient cells, leading to severe deformations of the nucleus, including decreased volume and increased thickness, as well as an increase in lateral bumpiness and roughness (Kim et al., 2017). The nuclear envelope lamina act as a “molecular shock absorber” thanks to a mesh of interconnected rods that are compressible to only a certain extend (Dahl et al., 2004).

IV-2-3. Cytoplasmic Intermediate filaments

IFs also act as a protector of the nucleus. Cytoplasmic IFs form a cage-like structure surrounding the nucleus, forming a lasso of filament around it. This structure offers protection to the nucleus and allows interaction between the cytosolic component and the nucleus (Méjat & Misteli, 2010). IFs are connected to via the crosslinker protein plectin to the cytoplasmic domain of nesprin 3 which then connects to the protein SUN of the inner envelope and to lamins IFs. Compressive stresses such as those induced by migration through confined spaces disrupt the integrity of the nucleus in absence of vimentin. The cage-like structure of vimentin around the nucleus gives the nucleus an additional layer of protection to maintain its integrity (Patteson, Vahabikashi, Pogoda, Adam, Goldman, et al., 2019). The anchoring of cIFs through the LINC complex allows them to transmit cellular events to the nucleus and adjust their positioning (Alam et al., 2016; Mellad et al., 2011).

IV-3. Cytoskeleton and nucleus positioning

The nucleus position within the cell changes and is associated with different biological processes depending on the cell type, the migratory state, the differentiation status or the stage of the cell cycle. For example, the nucleus is located at the rear of migrating cells, distanced from the protruding front (Gundersen & Worman, 2013; Maninová et al., 2013). Dysregulation of nuclear positioning leads to cellular dysfunction and disease as seen in Emery-Dreifuss muscular dystrophy or lissencephaly (Almonacid et al., 2019). To be moved, the nucleus needs an anchoring and cable pulling or forces pushing and turning it in the correct position. It was first proposed in 1997 that the cytoskeleton link the nucleus to the ECM to regulate the shape and position of the nucleus (Maniotis et al., 1997). The principal or to say most studied actors are actin and MT and their associated molecular motors (Gundersen & Worman, 2013). IFs involvement has not been extensively studied. However, they are known to participate indirectly in the nucleus positioning within the cell, and it has been demonstrated especially during polarisation for migration. Actin retrograde flow pushes the nucleus towards the rear of the cell during fibroblast and neuronal migration. At the same time, IFs are transported to the perinuclear area along with the actin retrograde flow in an asymmetrical manner. The network of IFs thus around the nucleus, allows actin to push the nucleus to the back of the cells. Depletion of IFs results in the alteration of the nucleus translocation without perturbing the actin retrograde flow, moving under and above the nucleus (Dupin et al., 2011). The interaction between the two networks is therefore important for nucleus positioning. There are two mechanisms at work. The first one, just described, does not require the direct coupling of the cytoskeleton to the nucleus but merely affect the position of the nucleus through the restriction of specific cytoplasmic location access (Y. L. Lee & Burke, 2018). It is considered passive. It can be done by modulating the density of the network, for example here IFs density around the nucleus and in front, giving propulsion forces to the actin retrograde flow. The second mechanism involves the direct application of force to the nucleus surface and therefore need nuclear anchoring of the cytoskeleton through the LINC complex. The interaction SUN/KASH domain-containing proteins are essential for nuclear migration thanks to their covalent binding via disulfure bond, appearing well adapted as force resistant coupling mechanisms (Razafsky et al., 2014; Sosa et al., 2012).

Nucleus positioning is also mediated by a linear array of LINC complex protein nesprin 2G and SUN2 associated with actin called TAN lines (Transmembrane Actin-Associated Nuclear Lines). They are lines of actomyosin network attaching the nucleus in a perpendicular manner (Luxton et al., 2011). This anchorage of the actin to the nuclear envelope and lamin A, allows

transmission of the forces generated by the actomyosin network to the laminA and therefore controlling nuclear movement as the actin network reorganises.

Note:

IFs form a cage like structure around the nucleus that constitute a protective layer. Accumulation of IFs around the nucleus is known to be involved in its positioning in polarized cells

IV-4. Control of nuclear morphology

Forces affect the nuclear envelope structure and with it the nucleus morphology. Nucleus morphology is tightly linked to the cell surrounding and the cytoplasmic cytoskeleton as isolation of the nucleus or disruption of said cytoskeleton leads to drastic changes in morphology. Force application directly on the isolated nucleus via the LINC complex protein nesprin1 resulted in nuclear local stiffening associated directly with the nucleoskeleton changes in laminA/C and their partner emerin. Emerin becomes tyrosine phosphorylated upon forces application on the LINC complex which induce a strengthening of the bind between laminA/C and the LINC complex. This tight association between the lamins and the LINC complex allows stiffening and might protect the nucleus integrity, preparing it for movement and positioning within the cytoplasm (Guilluy et al., 2014; Guilluy & Burridge, 2015). In a virtual cross-section of NIH 3T3 fibroblasts cell nuclei, on soft substrate, they appear rounder and increase stiffness leads to the flattening of the nucleus. These morphological effects disappeared with the disruption of the LINC complex and the inhibition of myosin, with all nuclei being rounded, suggesting that cytoplasmic cytoskeleton is affecting the morphology of the nucleus depending on the rigidity of the substrate (Lovett et al., 2013). When the cell and the nucleus are submitted to external forces, such as migrating through confined spaces, there is a reorganisation of the LINC protein nesprin-2 giant localising at the front of the nucleus through its actin domain,

allowing actin network contractility to pull on the nucleus for it to pass constriction, rendering nesprin-2 a specific mechanical force transmitter (Figure 34) (Davidson et al., 2020).

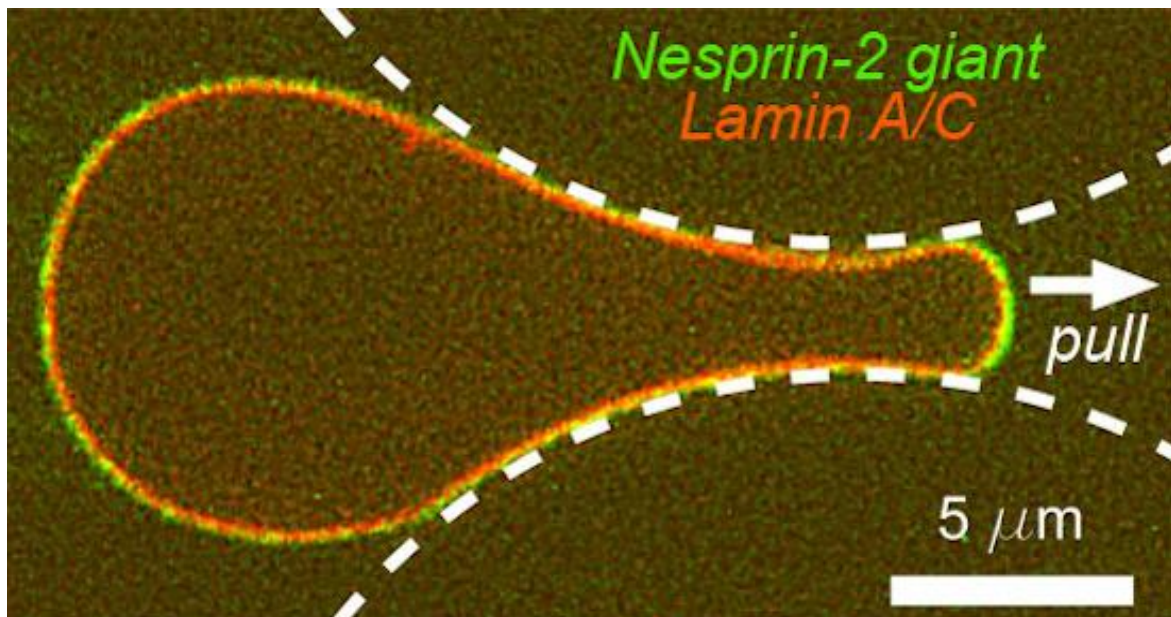


Figure 34 Nesprin accumulation in front of the nucleus in confined migrating cell. The outer nuclear envelope protein nesprin-2 is seen accumulating at the front of the nucleus when the cell migrates through narrow constrictions. Nesprin-2 links actin, therefore, actomyosin contractility can pull the nucleus through the constriction. (Davidson et al, 2020)

Nuclear rheology experiments helped to understand the extent of deformability of the nucleus upon cytoskeletal tension. Researchers can recreate the tension exerted on the nucleus by the cytoskeleton and measure the nuclear deformation. Upon short induced stress, the nucleus displays a more fluid-like behaviour, orchestrated by the reorganisation and the flow of chromatin. Long stretches and stress application increase the nucleus stiffness, with a more solid-like behaviour. This involves the lamin A/C that can stretch. A too long stress application leads to irreversible deformation (Mathieu & Manneville, 2019; Pajerowski et al., 2007). These findings are controverted by a study using TC7 isolated nuclei. Using microaspiration and AFM indentation, they found that nuclei of these cells resist deformation and stiffen at short times but deform and soften at longer times (Dahl et al., 2005). The behaviour of the nucleus upon mechanical stress might be different depending on what mechanical cues it is affected with. When compressing the nucleus between two plates and applying oscillatory stress, the nucleus becomes softer and more viscous at its periphery. This implies a decreased cross-link with chromatin at the periphery of the nucleus. It could well indicate that mechanical cues direct the reorganisation of the most active region of the nucleus to change gene expression (Lherbette et al., 2017). It is also important to note that the differential status of the cell is linked to the stiffness and viscosity of the nucleus. Indeed, in less differentiated cells, where gene expression

is highly active, the nucleus is more viscous and deformable, due to the high dynamism and activity of the chromatin. Once the cell is differentiated, the nucleus becomes less malleable to stress-induced deformation (Pajeroski et al., 2007).

IV-5. Mechanotransduction induces changes in gene expression

IV-5-1. Effects of mechanical signals on chromatin

Nuclear stiffness is indeed due to lamins but also chromatin structure. Chromatin is taking most of the volume of the nucleus as its viscoelastic properties change following the ratio of heterochromatin and euchromatin (Miroshnikova et al., 2017; Nava et al., 2020). This account for the stiffening of the nucleus and is mediated by gene regulation and PTM of histones. Chromatin primary function is to package the DNA in a condensed form around protein cores called histones.

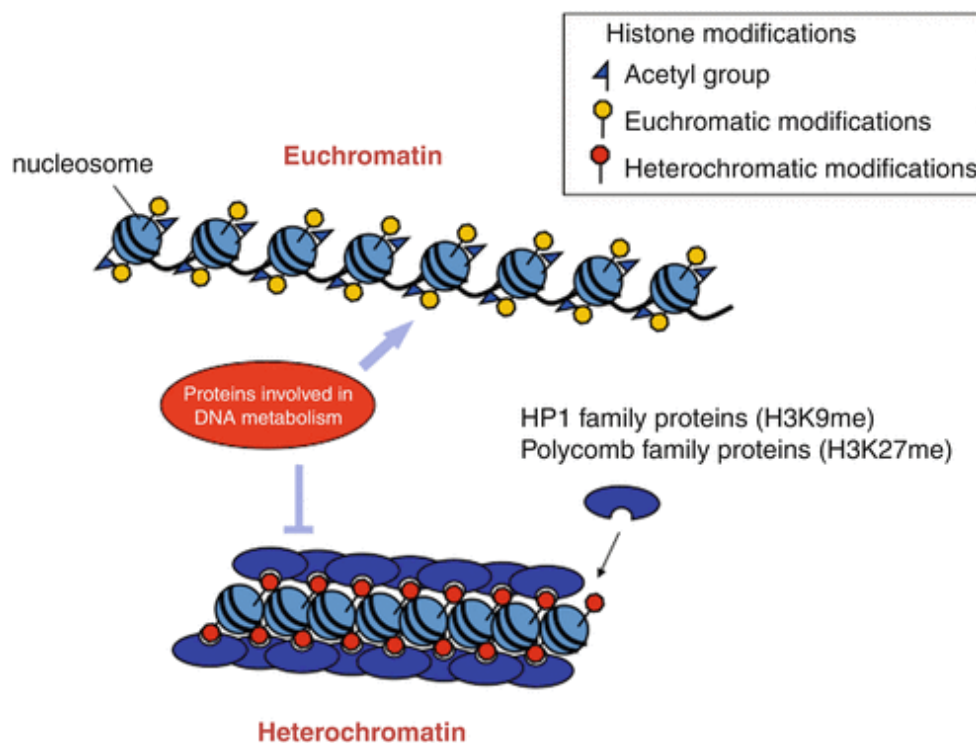


Figure 35 Different structure of the chromatin. Euchromatin is unpacked to allow higher gene expression, thanks to histone acetylation on the tail and heterochromatin is densely packed due to methylation of the core histones (Murakami, 2013).

The density of packaging of the complex DNA/histones called nucleosome determines the structure of the chromatin: heterochromatin is densely packed and euchromatin is loosely packed facilitating gene expression (Figure 35) (Murakami, 2013). The transition between heterochromatin and euchromatin is mediated via PTM of histone protein tails, principally acetylation and methylation of the four different kinds of histones (H1, H2A, H2B and H3) (DNA Packaging, n.d.). Mechanical stimuli lead to changes in chromatin states. A recent study investigating mechanical stretching of the isolated nucleus demonstrates the impact of

mechanical forces on chromatin. They found that chromatin is a major player in nuclear force response through the resistance to small deformation and has only a secondary role in large deformation (Stephens et al., 2017). They also found that the euchromatin/heterochromatin ratio affect the stiffness of the nucleus and is modulated upon stretching. Changes in chromatin structure upon mechanical stimulus lead to changes in gene expression.

Note:

Forces control nuclear morphology. Nucleus are flattening with increased stiffness. Changes in nuclear morphology can be promoted by a change in chromatin structure allowed by PTM of core histones.

IV-5-2. Control of gene expression in response to mechanical tension

Mechanical stimuli on the cell result indeed in intracellular changes tightly linked to gene expression and cytoskeleton regulation. These changes are only done through the mechanical impact on the nucleus that leads to gene regulation and expression (Chiquet et al., 2009), starting usually with transcription factors regulation, expression and translocation. The microenvironment is changing the fate, shape and development of the cell, by regulating specific downstream signalling pathways. The pathway regulated largely depend on the cell types but some common features are present. Among the principal signalling cascades following changes in gene expressions through mechanotransduction are firstly calcium-dependent signalling. In tendon and connective tissue, this signalling cascade has been observed through stretch-activated and voltage-activated calcium channels, such as Ca_v1 as well as gap junctions (Caluori et al., 2019; Lyford et al., 2002; Wall & Banes, 2005). When forces are applied to integrins complexes through collagen embedded beads, immediate calcium influx is observed ($<1s$). After force application, vertical extension of the cells was counteracted by an actin-mediated retraction. This leads to the accumulation of actin at the contact between the beads inducing a 6fold increase in membrane rigidity, dependent on calcium release and tyrosine phosphorylation. Repeated mechanical stress application progressively lessens the calcium flux and the cell adapts to the stress through actin cytoprotective properties (Glogauer et al., 1997; Matthews et al., 2006).

MAPK. The second type of signal transduction response after mechanosensing is the stimulation of the Ras family of small GTPase and the mitogen-activated protein kinases (MAPK). Indeed, in osteosarcoma cell lines and endothelial cells, after mechanical stress application, there is increased phosphorylation of MAPK. This suggests that mechanical induced regulation of gene expression is controlled by different activation of MAP and mediated by integrins (Ishida et al., 1996; Schmidt et al., 1998).

NF- κ B. Actomyosin influence in the cellular response to mechanical cues is omnipresent. They are also at the heart of the induction of the NF- κ B pathway. Indeed, NF- κ B, a transcription factor of extreme importance in immune reaction and cancer progression, is found to be mechano-regulated: NF- κ B activation is temporally induced in cells plated on stiff substrates and is not induced when cells are plated on soft substrate. Its activation, although integrins independent, is dependent on the phosphorylation of the myosin light chain inducing actomyosin contraction, resulting in morphological changes such as cell area expansion on stiff substrates (Ishihara et al., 2013).

Wnt/ β -catenin. The Wnt/ β -catenin pathway is crucial in development, stem cell differentiation and homeostasis. Its involvement and activation upon mechanical stress is becoming clearer. For example, Upon shear stress on the lymphatic vasculature, the pathway is triggered and in turn activate the lymphedema-associated transcription factor FOXC2 that promotes lymphatic vessel maturation (Cha et al., 2016). A study by Przybyla et al (2016) showed that softer environment (0.4kPa) induced the expression of Wnt3a in human embryonic stem cells, which in turn activate the Wnt/ β -catenin pathway (Przybyla et al., 2016). The softer environment is compliant with the embryonic gastrula tissue. Stiffer environments induce β -catenin degradation, inhibiting differentiation. It is also interesting to see that the Wnt/ β -catenin pathway is regulated by the Hippo pathway, described in the next paragraph. TAZ is binding to Dvl (dishevelled scaffolding phospho-protein from the Wnt pathway) that inhibits the Wnt/ β -catenin signalling (Varelas et al., 2010) and Dvl bind to phosphor-YAP changing its localisation (Y. Lee et al., 2018).

YAP/TAZ. Yes-associated protein (YAP) is a mechanosensitive transcriptional regulator from the hippo pathway that act as a mechanotransducer. Together with the transcriptional coactivator with PDZ-binding motif (TAZ), YAP sense and respond to mechanical cues leading to the regulation of gene expression (Totaro et al., 2018). Its main regulatory mechanism depends on its localisation in either the nucleus or the cytoplasm. Reduced number of adhesion and lower contractility observed in cells plated on soft rigidities deactivate YAP/TAZ pathway and YAP has a more cytoplasmic localisation. On the other hand, on stiff rigidities, increased actomyosin contractility and chemical cues, activate YAP and it translocates to the nucleus, where it can bind to the transcription factor TEAD (Dupont et al., 2011; B. Zhao et al., 2008).

Signalling cascade including the Hippo and Wnt pathway, as well as specific proteins such as integrins, FAK, Rac1, β -PIX, PAK and Src, can regulate YAP/TAZ dependent mechanotransduction (Aragona et al., 2013; Totaro et al., 2018). YAP translocation is also dependent on the coupling of the cytoskeleton to the nucleus via the LINC complex. Indeed, when the coupling is impaired, using LINC dominant-negative cells, the translocation of YAP in the nucleus in stiff substrates is no longer observed (Elosegui-Artola, et al., 2017). Coupling of the cytoplasmic cytoskeleton with the LINC complex leads to the opening of the nuclear pore when contractility is present, on stiff substrate facilitating the transport inside the nucleus and allowing YAP nuclear accumulation (Figure 36). Therefore, YAP translocation is an excellent readout, to see if the cell is reacting to the different rigidities of the ECM.

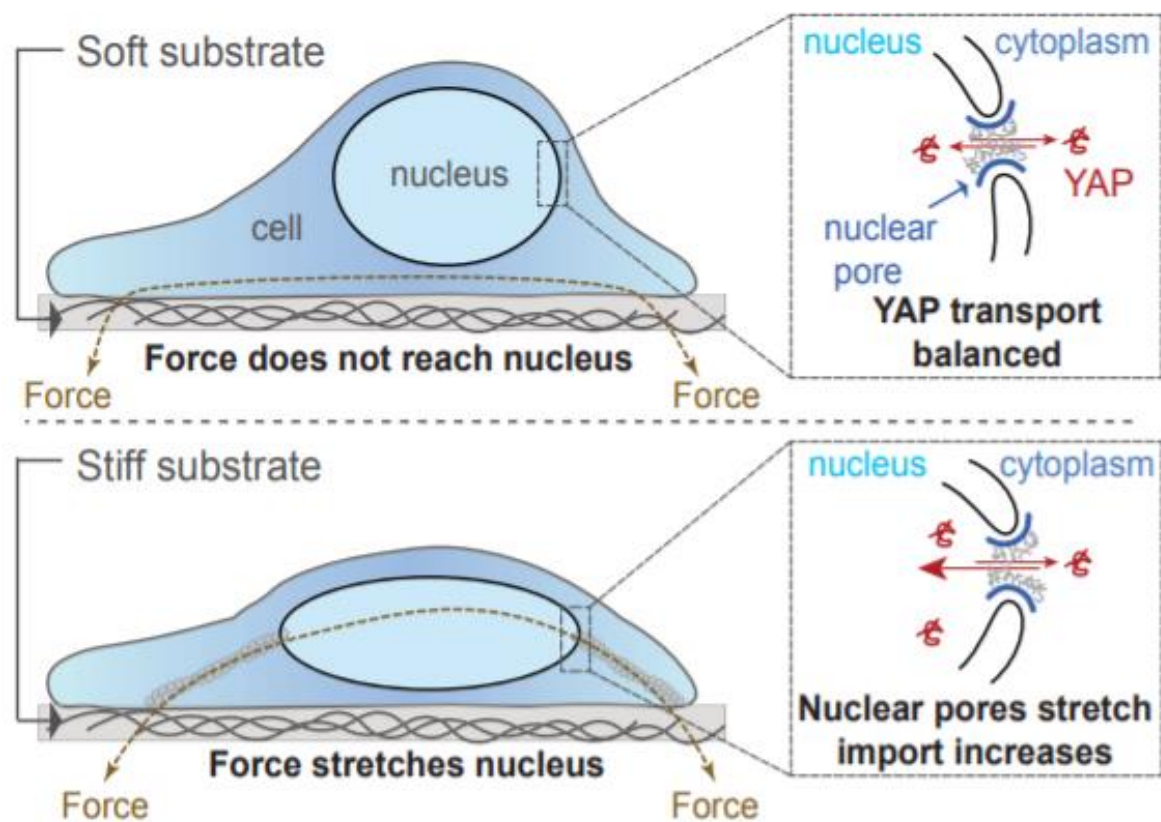


Figure 36 YAP enters the nucleus on stiff substrate. The actomyosin network flatten the nucleus on stiff substrate leading to the stretch of nuclear pores that allow the entry of YAP in the nucleus (Elosegui-Artola, 2017)

The YAP/TAZ pathway is closely linked to the MRTF-SRF (Myocardin Related Transcription Factor – Serum Response Factor) pathway in regulation cytoskeleton dynamic and tension. Both pathways are sensitive to the external mechanical stimuli. Expression of both pathway derivative protein can activate mutually activate, their cross-talk requires the recruitment at the DNA binding site facilitated through their DNA binding partner, TEAD for YAP and SRF for MRTF.

Both pathway interact indirectly through their ability to control the cytoskeletal dynamic (Foster et al., 2017).

In a disease state or when the cell is transformed, the capacity of the cell to sense the rigidity of the substrate and adapt can be seriously impaired or even abrogated. This might allow disease progression. Understanding fully the mechanism by which the cell are sensing the rigidity and the actors involved is, thus, crucial (Doss et al., 2020). This in turn gives us insight into the functional consequences of mechanotransduction on cell morphology and metabolism changes.



Translocation of YAP inside the nucleus is an excellent readout of mechanical tension exerted by the cell on the nucleus. With tension, the nuclear pores open and let YAP inside the nucleus.

IV-6. Key messages

- A. The nucleus is an important organelle in mechanotransduction, as it is tightly linked with the cytoskeleton. The cytoplasmic cytoskeleton is linking nuclear IFs, lamins, through the LINC complex. This complex allows anchoring of the nucleus and movement and shape changes.
 - B. IFs anchor the nucleus through plectin and nesprin-3 and its KASH domain, the cytoplasmic part of the LINC complex. IFs form a nuclear cage, which protects the nucleus from mechanical stresses.
 - C. Mechanotransduction at the nucleus allows changes in shape and affect gene expression. As an example, the transcription factor YAP translocates to the nucleus in stiff environments and activate specific gene expression.
- ➔ Whether IFs participate in mechanotransduction at the nucleus is still unknown, however their interaction and cage like structure strongly point towards its involvement in these pathways.

Objectives

V. Objectives

IFs participate in a myriad of cellular processes. IFs are known to be upregulated in glioblastoma. Astrocytes are a good and well-known model to study IFs in health and to understand their mechanical contribution to cell integrity, as IFs are highly elastic, apolar and resistant to tension.

A previous study in the lab highlighted the importance of IFs in the regulation of traction forces exerted by the actomyosin network on FAs. The absence of IFs led to an increase of traction forces on the ECM accompanied by a reduced FA turnover. However, very little is known about the direct changes of rigidity effect on IFs. The fact that IFs are interacting with FA proteins and are associated with actomyosin cables anchored at FAs, strongly suggest that substrate rigidity can affect the organisation and/or dynamics of cIFs.

Most notably, the IF network within the cell interacts tightly with the nucleus forming a cage-like perinuclear structure. IFs are forming a lasso around the nucleus, most likely anchored through the LINC complex interacting with plectin and nesprin-3. We thus hypothesised that IFs participate in the shaping of the nucleus.

The main question asked in this thesis is whether cytoplasmic IFs may contribute to nuclear responses to substrate rigidity.

To address this question, we axed this thesis into three different parts. Firstly, we will assess if substrate rigidity affects the nucleus morphology. To do so, we reproduced *in vitro* rigidities that the cell encounters *in vivo*. We will then study how IFs organisation, especially around the nucleus, is changed with rigidity. We focused on the role of IFs in the mediation of the changes that substrate rigidity has on the nucleus. We then further looked at the effect of geometry constrain on the nucleus, using micropatterns of different shapes and sizes. Finally, we looked for new binding partners of IFs and focused our attention on HDAC6, enzyme deacetylating primarily MTs.

V-1. What are the effects of mechanical cues on nuclear morphology and structure?

V-1-1. Does substrate rigidity affect the nucleus size and morphology?

The nucleus is a dynamic organelle, able to move around the cell during cellular activity such as cell migration. It is thought to be a primary actor in the mechanosensitive pathways. Its size, morphology, position has been shown to change in contact depending on substrate rigidities. We focused the first part of the work to see if substrate rigidity could affect the nuclear size, morphology, volume and positioning in astrocytes.

V-1-2. Does substrate rigidity affect the chromatin status?

We wanted to assure that our model was responsive to changes in rigidity and thus changes in internal tension, and if this tension was affecting the gene expression by looking specifically at the conformation of the chromatin. Changes were assessed by quantifying the expression of Histone PTM. Acetylation of histones was used to assess the euchromatin, and methylation H3K9me3 to assess the heterochromatin status.

V-1-3. Does substrate rigidity affect the nuclear recruitment of transcription factor YAP?

To assess if astrocytes can well sense and respond to the different tension exerted by the substrate rigidity, we looked at the nuclear recruitment of YAP. Accumulation of YAP in the nucleus account for higher tension exerted by the cytoskeleton on the substrate.

V-1-4. Does geometrical constrain affect the nucleus morphology?

When cells try to fit in a physically restraining environment, their cytoskeleton is exerting a lot of internal forces to adapt and spread into adequate shape. Using micropatterns of different shapes and widths, we assessed how the nucleus is changing in size and morphology, to make a parallel with the substrate rigidity. We looked at the integrity of the nucleus and its facility to fit and change morphology on narrow patterns. Results are presented in the appendix.

V-2. Is the IF network reorganised in response to mechanical cues?

V-2-1. Does IF organisation change with the substrate rigidity?

IFs are present in different forms within the cell. A soluble pool of tetramers, ULF, squiggles and filaments. They can associate and dissociate and change organisations with different cellular events. For example, IFs is phosphorylated and therefore made soluble during cell division. They polarise the network towards the wound edge during migration. Substrate rigidity was shown to influence vimentin IF phosphorylation and solubility in fibroblasts. The organisation of the IF network is likely to dramatically affect its mechanical and scaffolding functions. We wanted to understand if the substrate rigidity modifies the organisation of the network.

- IFs are known to form a cage-like structure around the nucleus, however, it was only described in stiff substrates. IF proteins being transported towards the nucleus through the retrograde flow of actin, and actin changing conformation on soft rigidities, we focused this part on the characterisation of IF cage-like structure around the nucleus on different rigidities.
- If changes occurred, we looked at the phosphorylation status to characterise any changes in solubility of the network on the different rigidities.

V-3. Do IFs mediate the effect of substrate rigidity on the nucleus?

IFs extend between FAs and the nucleus. They are in direct contact with the nucleus and form a cage around it. We showed in a previous study that IFs are controlling the actin driven traction forces at FAs. As well as the nuclear actin-driven nuclear positioning. It is interesting to understand if this control is exerted upstream at the nucleus itself. We thus hypothesized that IFs could participate in the mechanosensitive responses of the nucleus. To test this hypothesis, we used siRNA to knockdown IFs and assessed if changes of the nucleus shape and size but also gene expression regulation by substrate rigidity was affected by the lack of IFs. If changes occur when the cell is depleted of IFs, it would suggest that IF participate in the nuclear response to substrate rigidity.

V-4. Looking for effectors of IFs new partners

We looked for new binding partner of IFs through semi quantitative mass spectrometry. We looked at specific interactors of IFs with the nucleus and the other cytoskeletal component, and especially partners that could potentially be mechano-regulated, or involved in the mechanotransduction pathways.

Material and Methods

VI. Material and methods

VI-1. Cell culture:

Primary Astrocytes are cultured from OFA rat embryos. The temporal lobes free from meninges are dissected and mechanically dissociated. The cell suspension is plated onto P-100 Polyornithin coated Petri dishes for seven days to select for astrocytes. Other cells and debris are thoroughly washed with PBS 7 days later. Astrocytes are grown in 1g/L glucose DMEM Glutamax (Gibco) supplemented with 10% FBS (Invitrogen), 1% penicillin-streptomycin (Gibco), and 1% amphotericin B (Gibco) at 37°C, 5% CO₂.

HEK cells were cultured in 4gxL⁻¹ glucose DMEM Glutamax (Gibco) supplemented with 10% FCS (Invitrogen), 1% penicillin-streptomycin (Gibco), and 1% amphotericin B (Gibco) at 37°C, 5% CO₂.

VI-2. Transfection:

Astrocytes were transfected with DNA mixed with Lonza® glial transfection solution and electroporated using an Amaxa® nucleofector machine. HEK cells were transfected with the calcium chloride technique: 2µg of DNA was mixed with a solution containing 90% water and 10% 10x CaCl₂ solution for 20min at RT. The solution was then incubated with the same volume of HBS at RT for 13min, before being added to the freshly passed HEK cells. Cells were ready to use 3 to 4 days post-transfection.

Si RNA sequences used:

- Luciferase (control) : UAA GGC UAU GAA GAG AUA C
- Vimentin rat: UGA AGA AGC UGC ACG AUG A
- GFAP rat: GAG UGG UAU CGG UCC AAG U

Plasmid used:

- Vimentin peGFP N3
- Δ-GFAP peGFP N3,
- Nestin peGFP N3
- HDAC6-peGFP.

VI-3. Hydrogel substrates of different rigidities.

Hydrogels are made to generate an extracellular matrix of controlled and reproducible rigidities. Two protocols were used: one with ranging rigidities from 2kPa to 200kPa (See table figure 2) and a second one from 0.2kPa to 48kPa. Bottom coverslips are plasma cleaned for 3min and then silanised for 10 min in a solution of 1% acetic acid and 1% silane in absolute ethanol. This step will allow the gel mixture to attach to the coverslip. After ethanol washes, gels mixtures are

left to polymerise between two coverslips, top coverslip micropatterned or glass, for one hour at RT in a dark chamber. The gel surface is then activated with sulfo-sampah for 5min in a UV chamber and coated with fibronectin (1/100 in PBS) at 37°C for 1h. Cells are plated onto the gels overnight and fixed in the morning.

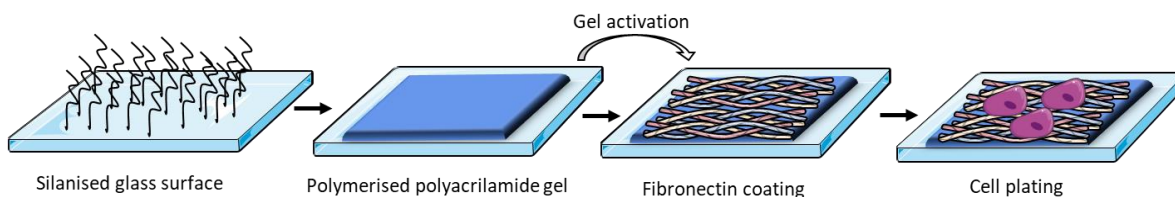


Figure 1 Fabrication of polyacrylamide gels

Young Modulus (kPa)	200	100	25	10	5	2
40%Acrylamide (μL)	125	125	125	125	125	125
2%Bis acrylamide (μL)	200	100	25	12.5	5	2.5
HEPES (μL)	175	275	350	362.5	370	371.5
%Acrylamide	10	10	10	10	10	10
%Bis acrylamide	0.8	0.4	0.1	0.05	0.02	0.01



Add:

- 2.5 μL of APS
- 0.25 μL of TEMED
- 25 μL of the solution onto the coverslips

Figure 2 Table of the volume needed to prepare the gels. Volume for a 500 μL solution

VI-4. Micropatterns

Glass coverslips (18x18mm) are plasma cleaned for 3 min and coated with 0.1 mg/ml poly-L-lysine/polyethylene glycol (PLL-PEG) diluted in 10mM HEPES for 30 min at RT. Hydrophobic coverslips are dried with milliQ water to remove the excess PLL-PEG and micropatterns printed with a chrome mask with ring, crossbow, disk and quadrant patterns. The printed coverslips are then coated with 2% Fibronectin solution (Sigma) diluted in 8.4g.L⁻¹ NaHCO₃ for 30min at RT and then washed with NaHCO₃. Cells are allowed to adhere overnight before fixation.

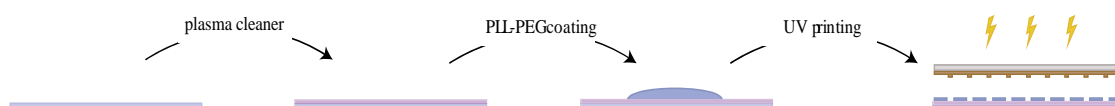


Figure 3 Micropatterning technique, scheme by Shailaja Seetharaman

The micropatterning form on the mask were designed using the autocad[®] software. Several forms were designed by hand: Crossbow patterns, round, lines of different widths (5, 10, 25 μ m) and square and rectangle same area (50x50, 25x100, 10x250, 5x500 μ m). The mask design was then sent for production to the company Toppan[®].

VI-5. Immunofluorescence

Astrocytes were fixed in 4% paraformaldehyde at 37°C for 10min and then permeabilized with 0.1% Triton X-100 for 5min at room temperature (RT) or fixed and permeabilised with cold methanol for 3min at -20°C. The cells are then blocked for 45 min in 5% Bovine Serum Albumin in PBS at RT. Primary and secondary antibodies diluted in 5% BSA are incubated for 1h at RT. After PBS washes and 5s incubation in Hoechst containing water, coverslips are mounted with Diamond Prolong Gold mounted medium. Epifluorescence images are acquired with a Leica DM6000 microscope with 40x1.25-NA or 63x1.4-NA oil objectives.

VI-6. Immunoprecipitation:

Confluent P-100 Petri dishes of GFP-tagged proteins (Vimentin, Nestin, GFAP and HDAC6) transfected HEK cells are lysed with 1% lysis buffer (25 mM Tris HCl, pH 7.5, 1mM EDTA, pH 8, 150mM NaCl, 1 mM MgCl₂ and 0.5% Triton X-100) freshly supplemented by protease inhibitors cocktail and 10% phosphatase inhibitors. The lysate is centrifuged at 100g for 10min. 20 μ l of the supernatant is stored as input in Nupage blue supplemented with 10% DTT at -20°C. To prevent MT and actin coprecipitation respectively, Nocodazole (1%) and Latrunculin (1%) are added and left 10min at RT. The IP is then performed following the Chromotek[®] protocol. After washing the beads three times with washing buffer (50mM Tris, pH 7.5, 150mM NaCl, 1mM EDTA, pH 8, 2.5mM MgCl₂), the sample is boiled in Nupage blue for 10min to allow separation of the immunocomplexes. The beads are pelleted and the supernatant loaded into a precast gel (Invitrogen[®]) for Coomassie analysis or Western Blotting. 5 μ L of the beads are sampled before separation and mounted on coverslips in Prolong Gold mounting media for GFP fluorescence visualisation.

For mass spectrometry analyses, the IP products were washed three times in ammonium solution right after the last three washes. The samples were then stored overnight at 4°C to be sent to the mass-spectrometry facility the next day.

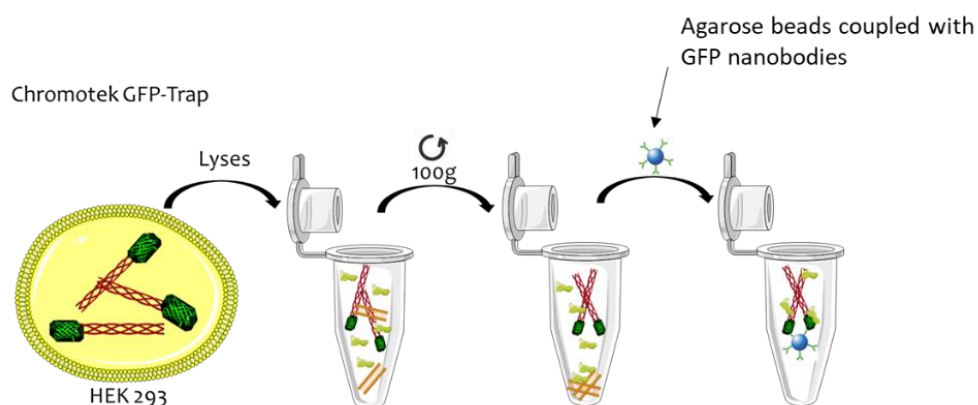


Figure 4 Immunoprecipitation using chromotek technology, adapted for IFs.

Co-sedimentation of Vimentin and HDAC6 protein was achieved by polymerising the vimentin in KCL, and mixing the polymer with purified HDAC6 proteins for 1h at 4°C and then centrifuging the solution for 30min at 20000g.

VI-7. Mass spectrometry

Proteins on beads were washed twice with 100 μ L of 25 mM NH_4HCO_3 and we performed on-beads digestion with 0.2 μ g of trypsin/LysC (Promega) for 1 h in 100 μ L of 25 mM NH_4HCO_3 . Samples were then loaded onto a homemade C18 StageTips for desalting. Peptides were eluted using 40/60 MeCN/ H_2O + 0.1% formic acid and vacuum concentrated to dryness. Online chromatography was performed with an RSLCnano system (Ultimate 3000, Thermo Scientific) coupled to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Scientific). Peptides were trapped on a C18 column (75 μ m inner diameter \times 2 cm; nanoViper Acclaim PepMapTM 100, Thermo Scientific) with buffer A (2/98 MeCN/ H_2O in 0.1% formic acid) at a flow rate of 4.0 μ L/min over 4 min. Separation was performed on a 50 cm \times 75 μ m C18 column (nanoViper Acclaim PepMapTM RSLC, 2 μ m, 100 \AA , Thermo Scientific) regulated to a temperature of 55°C with a linear gradient of 5% to 25% buffer B (100% MeCN in 0.1% formic acid) at a flow rate of 300 nL/min over 100 min. Full-scan MS was acquired in the Orbitrap analyzer with a resolution set to 120,000 and ions from each full scan were HCD fragmented and analyzed in the linear ion trap. For identification, the data was searched against the Homo sapiens (UP000005640) SwissProt database using SequestHF through proteome discoverer (version 2.2). Enzyme specificity was set to trypsin and a maximum of two missed cleavage site were allowed. Oxidized methionine, N-terminal acetylation, and carbamidomethyl cysteine were set as variable modifications. Maximum allowed mass deviation was set to 10 ppm for monoisotopic precursor ions and 0.6 Da for MS/MS peaks. The resulting files were further processed using myProMS 45 v3.6 (work in progress). FDR calculation used Percolator and was set to 1% at the peptide level for the whole study. The label free quantification was performed

by peptide Extracted Ion Chromatograms (XICs) computed with MassChroQ version 2.2 46. For protein quantification, XICs from proteotypic peptides shared between compared <conditions (TopN matching) with no missed cleavages were used. Median and scale normalization was applied on the total signal to correct the XICs for each biological replicate. To estimate the significance of the change in protein abundance, a linear model (adjusted on peptides and biological replicates) was performed and p-values were adjusted with a Benjamini–Hochberg FDR procedure with a control threshold set to 0.05. Up-regulated proteins with at least 3 proteotypic peptides (fold change > 1.5 and p-value < 0.05) and the unique proteins identified only in the GFP- α TAT1 were used for gene ontology (GO) enrichment analysis by using GO::TermFinder tools (10.1093/bioinformatics/bth456) through myProMS (Seetharaman et al., 2020).

VI-8. Western Blotting

Cell lysate and immunoprecipitation solutions were run by western blot for protein quantification. After being boiled for 10min, the solutions were loaded onto precast Invitrogen gels and run at 110V for approx. 1h30 to allow protein separations. Proteins are then transferred from the gel to a nitrocellulose membrane (Biorad) at 100V for 1h or 0.05A overnight. Red Ponceau is then added to the membrane to visualise transferred proteins. The membrane is then incubated in 5% Milk solution in TBST for 1h, then in Primary antibody (See table) for 1h at RT or overnight at 4°C with constant agitation. After three times washes in TBS 0.1% Tween, Membranes were incubated in secondary antibody coupled with HRP from Jackson ImmunoResearch for 1h in agitation at RT. Protein bands were revealed using Biorad ECL solution inside a chemiluminescence machine (Chemidoc Biorad).

<i>Antibody against</i>	<i>Company</i>	<i>Reference</i>	<i>Dilution</i>
<i>GFAP</i>	Dako	Z0334	WB 1/1000; IF 1/500
<i>GAPDH</i>	Chemicon	MAB374	WB 1/1000
<i>Vimentin</i>	Santa cruz	Sc-6260	WB 1/1000; IF 1/700
<i>HDAC6</i>	Abcam	Ab253033	IF 1/400
	Cell signaling	7612S	WB 1/500
<i>GFP</i>	Novusbio	NB600-313	WB 1/5000
<i>AcH2B</i>	Cell signaling	12799S	WB 1/1000; IF 1/500
<i>Nestin</i>	Millipore	MAB353	WB 1/500 IF 1/200
<i>AcH3</i>	Cell signaling	96349S	WB 1/1000; IF 1/500
<i>H3K9me3</i>	Cell signaling	13969S	WB 1/1000 IF 1/700
<i>A-tubulin</i>	Abcam	Ab4074	WB 1/1000; IF 1/500
<i>B-actin</i>	Sigma	A2228	WB 1/ 500
<i>YAP</i>	Sigma	Sc101199	IF 1/500
<i>Phalloidin</i>	JacksonImmunoResearch	715-295-150	IF 1/1000
<i>Vimentin phospho-S38</i>	abcam	Ab52942	WB 1/1000
<i>Vimentin phospho-S55</i>	abcam	Ab22651	WB 1/1000

Figure 5 List of antibodies used and their concentration

VI-9. Image analysis

Intermediate filament distribution was analysed with a custom-made macro in Fiji (See script in appendix 3). The contour of the nucleus was determined using the magic wand tool, and perinuclear intensity was calculated by incrementing 2.5 μm ring around the nucleus, as well as the total area of the cell and the intensity at the periphery of the cell, and the intermediate space. The ratio was calculated by dividing the mean fluorescence intensity in the perinuclear 2.5 μm ring by the mean fluorescence intensity at the middle of the cell, which represent the total fluorescence subtracted by the nucleus plus the 2.5 μm ring and the peripheral ring of 5 μm .

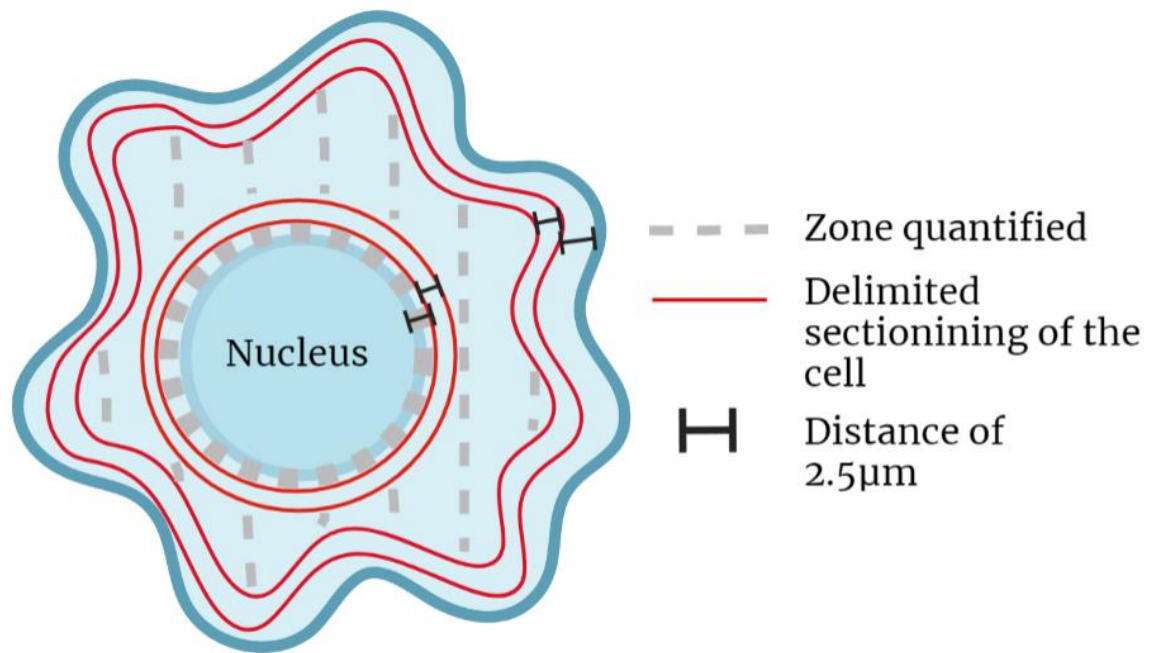


Figure 6 Scheme representing the region of the cell used in the quantification of IFs around the nucleus. The red region represents the segmentation by the macro, and great dotted line the region of interest used.

For the analysis of YAP localisation, nuclear localisation was analysed using the contour of the nucleus determined by the wand tool in fiji and cytoplasmic fraction determined by subtracting the nucleus area to the total area and measuring the mean of fluorescence. The ratio was then calculated dividing the nucleus mean intensity by the cytoplasmic mean intensity. For hetero and euchromatin, the total fluorescence intensity was quantified using the contour of the nucleus with DAPI and measuring the raw integrated intensity of the acH2B or H3K9me3 channel.

Volume analyses of the nucleus were done using the icy software and a protocol created in collaboration with the image analyses hub by Stephane Rigaud (See script in Appendix 4). Using confocal Z-stack of nucleus stained with Dapi or Hoechst, automatic segmentation of the shape is done and volume is calculated.

VI-10. Quantification and statistical analyses

All data are presented with the mean and +/- standard deviation (SD) of at least, otherwise specified, 3 independent experiments. Statistical differences between conditions were evaluated using student t-test or one-way ANOVA (ANalysis Of Variance) followed by a Tukey's post-hoc test. The analysis was done on the GraphPad Prism 6.0 software. Non-significance (p -value > 0.05) is assumed when not displayed, significance is shown for p -values as “*”: $p < 0.05$, “**”: $p < 0.01$, “***”: $p = 0.0001$; “****”: $p < 0.0001$.

Results

VII. Results

VII-1. Substrate rigidity affects the nucleus of astrocytes.

To study the role of substrate rigidity on the nucleus, we cultured primary rat astrocytes on previously described polyacrylamide gels of different rigidities with a range going from 2kPa to 200kPa (Judokusumo et al., 2012; Pelham & Wang, 1997; Wang & Pelham, 1998). The different substrate rigidity of polyacrylamide gels helped to model *in vitro* the substrate rigidities that astrocytes may encounter *in vivo*. We focused on the difference between 2kPa and stiffer substrates, due to the physiological rigidity of the brain being around 2kPa. Stiffer substrates would represent structures such as blood vessels or even represent the increased stiffness observed in pathological conditions as in the tumour environment. The cells were fixed and stained with Dapi or Hoechst to mark for DNA and ultimately the nucleus itself.

VII-1-1. Substrate rigidity affects the size and shape of the nucleus but not its position within the cell

We first showed that substrate rigidity affects the 2D areas of the nucleus. A significant increase in 2D area between all the steps of the rigidity range was observed with epifluorescence microscopy (Figure 1A). Here in cyan is staining of actin to show the contour of the cell. The nucleus appeared smaller on soft substrates and increased in size on higher rigidities. We then assessed the cell area to understand if the smaller cells were the cause of the smaller nucleus. However, we did not find a significant difference with the increased rigidity, nonetheless cells in 2 kPa tend to spread less and be slightly smaller. It was therefore important to assess how the nucleus behave in 3D. As previously shown in other cell types, substrate rigidity affects the shape of the nucleus, by flattening it (Lovett et al., 2013). The nucleus of our model, astrocytes, in 3D appeared rounder on 2kPa gels and seemed to flatten regularly with increasing rigidity when looking at confocal cross sections of Dapi stained nucleus on a confocal microscope (Figure 1D). However, by measuring the volume of the nucleus from z- stack images acquired with confocal microscopy, thanks to a macro that we developed with the software icy, allowing batch images processing, we found that the volume of the nucleus was significantly increased between 2kPa and 100kPa, same trend as for the 2D area.

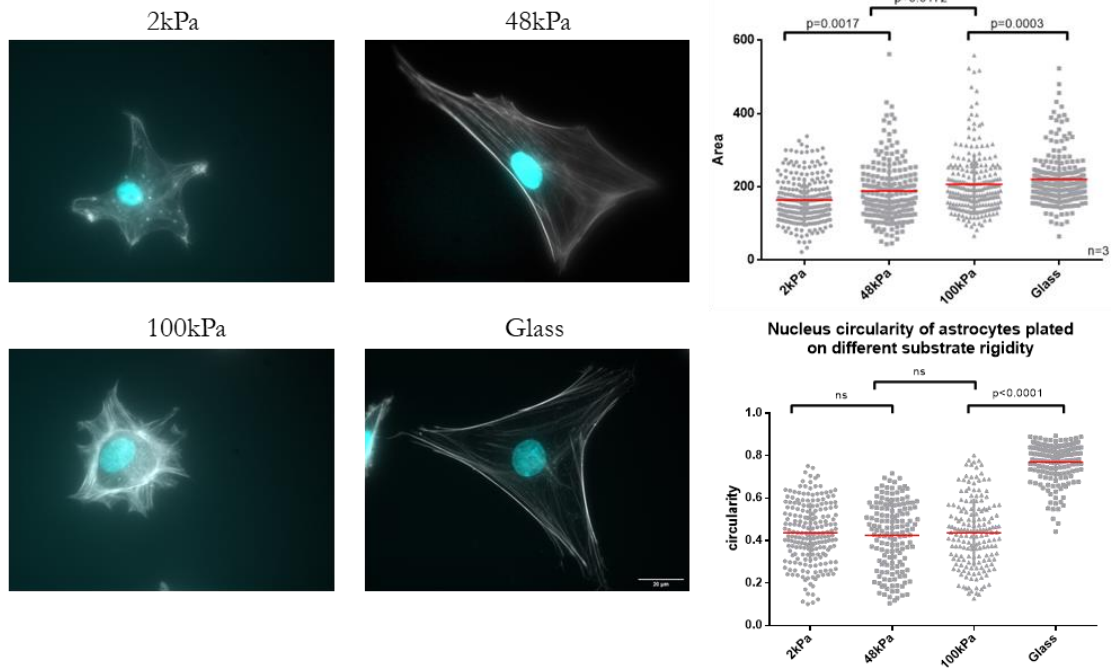
We then hypothesised that the tension needed to flatten the nucleus was affecting its shape and especially circularity. Circularity is measured with the formula $\text{circularity} = 4\pi(\text{area}/\text{perimeter}^2)$. A score of 1 would represent a perfect circle, and a score approaching 0, a very elongated polygon. We found that the different rigidities of the gels (2, 48 and 100kPa) did not affect the circularity of the nucleus in 2D, having a circularity score of 0.42 on average in Figure 1A. Nonetheless, higher circularity was found in glass where rigidity is immensely higher, the score

averaging at 0.8. This result suggests that substrates at least above 100kPa are needed to affect the circularity of the nucleus.

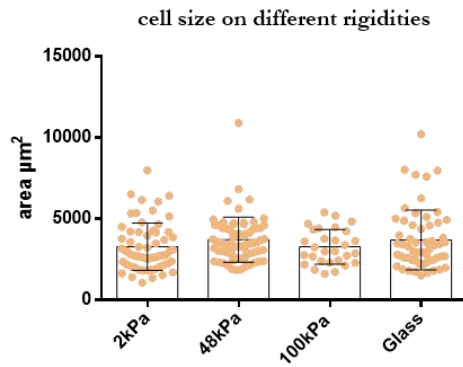
The cytoskeleton is responsible for the tension exerted on the nucleus (Alisafaei et al., 2019; Balakrishnan et al., 2020; Chancellor et al., 2010). Therefore, it can control the nuclear position within the cell (Dupin et al., 2011). The organisation of the cytoskeletal network was changed with rigidity (Introduction Figure 29), and we wanted to see whether the positioning of the nucleus was dependent on rigidity. On glass and sparse cells, it is usually centred. Therefore, we calculated the nucleus distance between the centre of the nucleus and the centre of the cell. However, the position did not vary within the cell, the nucleus remaining approximately centred, at an average displacement of $8\mu\text{m}$ displacement (Figure 1C). The changes in the actin organisation did not affect the positioning of the nucleus.

To sum up, nucleus of astrocytes on soft substrate are centred, smaller and rounder and on stiffer substrate, the nucleus appears centred, larger and flatter.

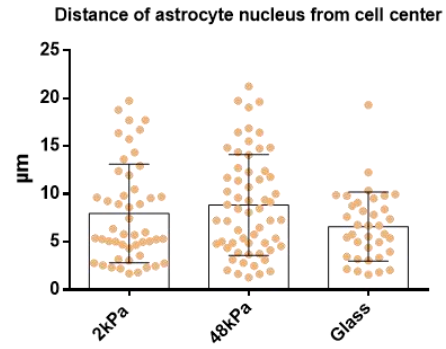
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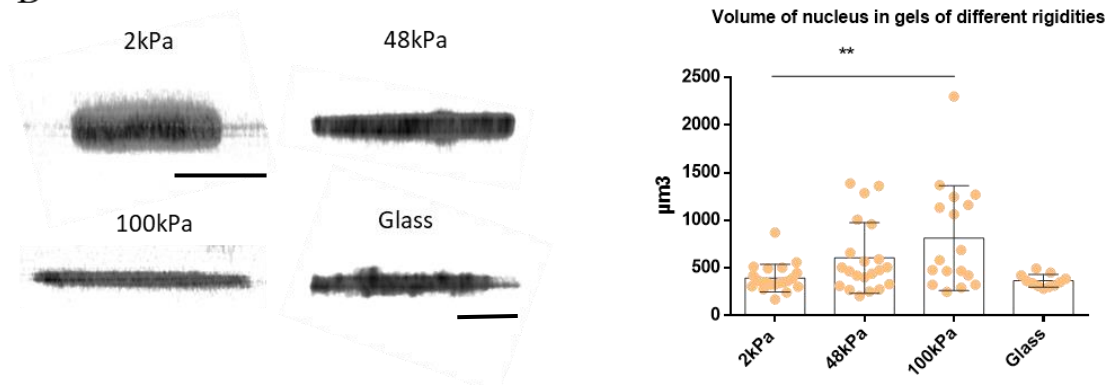


Figure 1 Substrate rigidity affect the nucleus morphology but not its position within the cell. (A) Representative epifluorescence images of astrocytes plated on different rigidity (2, 48, 100 kPa and Glass) with actin (gray) and the nucleus (cyan) scale bar= $20\mu\text{m}$. Graphs representing the mean (\pm SD) area of the nucleus in μm^2 (top) and the circularity score of $N=3$ independent experiment. (B) Graphs representing the mean (\pm SD) area of the cell in μm^2 (C) Graph showing the mean displacement between the centre of the cell and the centre of the nucleus of astrocytes plated on 2, 48 kPa and Glass $N=3$. (D) confocal z -stack 3D rendering of nucleus of astrocytes plated on different substrate rigidities. Image representative of $N=2$. Graph showing the volume of confocal microscopy z -stacks of the nucleus on different rigidities., $N=2$. Scale bar= $5\mu\text{m}$ Statistical analyses done using One-way ANOVA followed by Tukey's multiple comparison test (** $p<0.01$) (A-C).

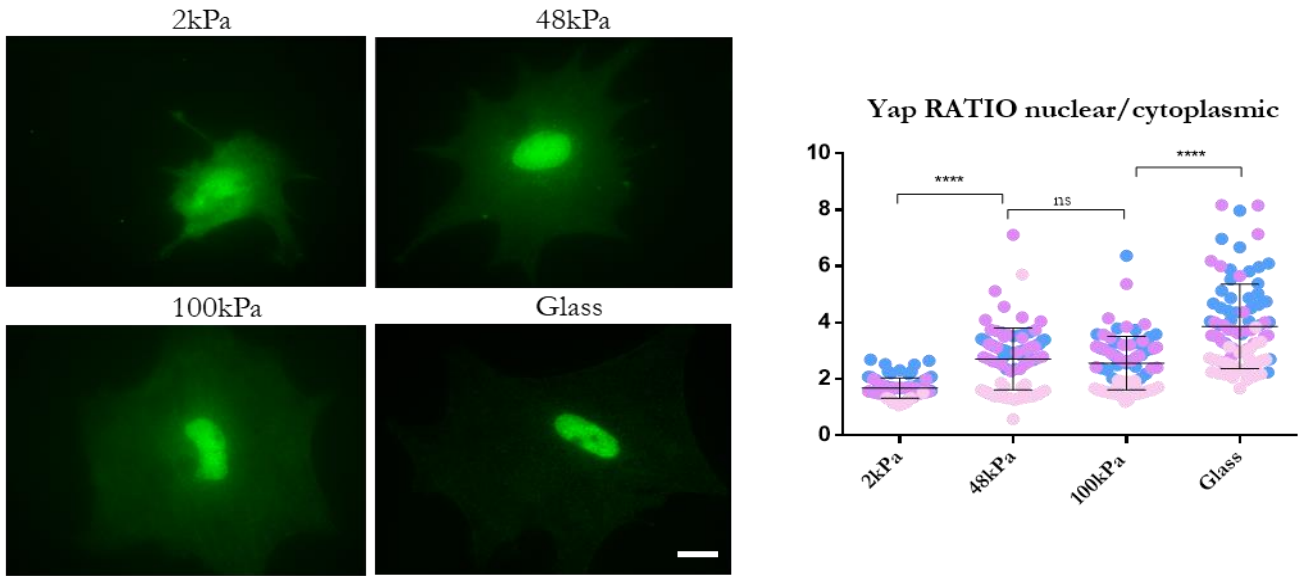
VII-1-2. YAP nuclear localisation in astrocytes

The flattening of the nucleus by the actomyosin network, that we observed, leads to the opening of the nuclear pores. In turn, this decreases the nuclear resistance to molecular transport (Elosegui-Artola, 2017). A very good readout to detect this phenomenon is the translocation of the transcription factor YAP to the nucleus. To test if the astrocytes were indeed sensitive to these changes in tension, we stained for YAP and quantified its localisation on different rigidities (Figure 2A). We did observe increased nuclear recruitment of YAP when cells were plated on stiff substrates, looking at the ratio between nuclear and cytoplasmic YAP. The ratio suggests that even on 2 kPa there was still nuclear localisation (ratio=1.8). The ratio, however significantly jumped to 2.4 on 48 kPa, plateauing for 100 kPa, and jumped again to 4 for the glass (Figure 2A). This increase in YAP nuclear localisation with rigidity confirmed that primary rat astrocytes are mechanosensitive. YAP being a transcription factor, we hypothesised that changes in gene expression could be observed between soft and stiff substrates.

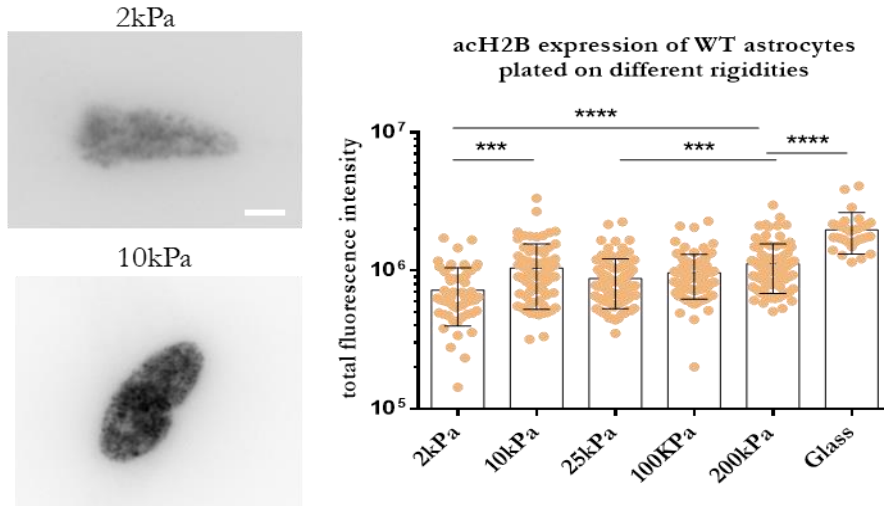
VII-1-3. Substrate rigidity affects histone post translational modifications

To study whether gene expression was indeed affected by substrate rigidity, we investigated changes in chromatin conformation using antibodies targeting acetyl histone H2B, a marker for euchromatin, and H3K9me3, a marker for heterochromatin and repression of gene activation. With the opening of nuclear pores with higher rigidity and the facilitation of transport to the nucleus, the chromatin should be in a more open conformation on stiff substrates. A significant increase in acetyl histone H2B was observed between 2 kPa and 10 kPa, plateauing at 100 kPa and surprisingly significantly increasing observed between 200 kPa and glass (Figure 2B). There is in total a 35.44% increase of acH2B expression between 2 and 200 kPa. A higher fluorescence intensity was observed at 10 kPa compared to 2 kPa as seen in Figure 2B. This was accompanied by a significant decrease of H3K9me3 between 2 kPa and 25 kPa, which correlate well with a change to more open chromatin (Figure 2C). The significant decrease between 2 and 200 kPa was valuated at 22.86%. These results suggest that gene expression increased in fact with rigidity between 2 and 100-200 kPa. However, an increase in H3k9me3 was observed between 100 kPa and glass.

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B



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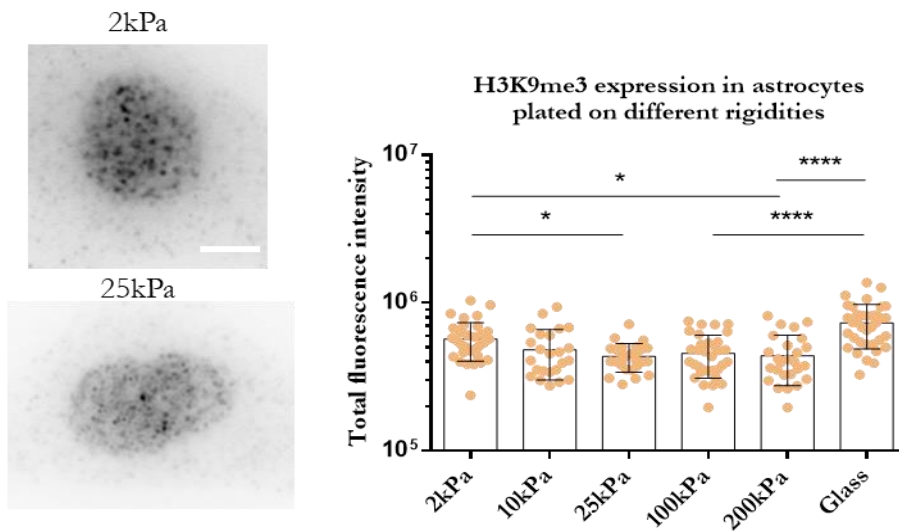


Figure 2 (previous page) Substrate rigidity affect the nuclear localisation of YAP and the conformation of the chromatin. (A) Representative images of Epifluorescence images of WT astrocytes on 2, 48, 100 kPa and Glass, stained for YAP (green) scale bar = 10 μ m. Graphs showing the mean ratio (+/- SD) of Yap in the nucleus by Yap located in the cytoplasm. Different colours represent N=3. (B) Inverted epifluorescence images of acH2B stained nucleus of WT astrocyte on 2 and 10 kPa, darker staining represent high epifluorescence intensity. Graph showing the difference in total intensity of acH2B within the nucleus on a range of rigidity (2, 10, 25, 100, 200 kPa and glass) N=3. Scale bar: 5 μ m (C) Inverted epifluorescence images of H3K9me3-stained nucleus of WT astrocyte on 2 and 25 kPa. Graph showing the difference in total intensity of acH2B within the nucleus on a range of rigidity (2, 10, 25, 100, 200 kPa and glass) N=3 Scale bar: 5 μ m. Statistical analyses done using One-way ANOVA followed by Tuckey's multiple comparison test (* p <0.05; *** p =0.0001; **** p <0.0001).

To summarise, on soft substrate, the nucleus appeared round and small, elongated, with YAP localising in the cytoplasm and the nucleus, and a more closed conformation of the chromatin, indicating a lower gene expression activity. On stiff substrates, the nucleus appeared flat and big, more circular in extreme rigidity, with YAP exclusively located in the nucleus, and possessing a more open chromatin conformation, suggesting a potential higher gene expression.

VII-2. IFs organisation is changing with the rigidity of the substrate

VII-2-1. IFs form a cage-like structure around the nucleus on stiff substrates.

To study the impact of substrate rigidity on the IF network, we seeded sparse astrocytes in different rigidities. The cells were left overnight and then fixed with PFA and stained for Vimentin, GFAP or Nestin and DAPI. We developed a fiji macro for quantification of the amount of IF fluorescence within a 2.5 μ m perinuclear ring using epifluorescence microscopy images of IFs and the nucleus on the different rigidities (script in appendix 4). On stiff substrates, here 100 kPa, vimentin was concentrating around the nucleus, forming a lasso of filament around it. It separated it and followed its exact shape (Figure 3A). On softer substrates as seen here at 10 kPa, vimentin was found to be more dispersed throughout the cytoplasm. Vimentin on 2 kPa is still slightly accumulating around the nucleus with a mean of 1.989 for the ratio of perinuclear and middle of the cell vimentin fluorescence. However, it increased significantly at 100 kPa with a mean of 4.006, corresponding to a 50.37% increase (Figure 3A, bottom panel). These results showed that stiffer substrates promote the formation of an IF nuclear cage.

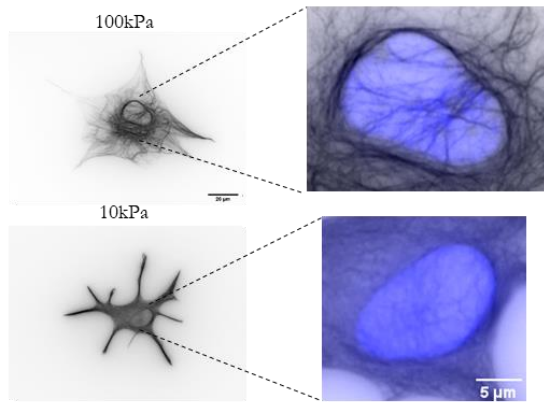
We performed the same analyses on Nestin, we also found an increase with rigidity, although more subtle than for vimentin: the ratio increased significantly from 0.7644 at 2kPa to 1.228 at 100 kPa, which corresponds to a 37% increase (Figure 3B). This suggests that vimentin may be relatively more enriched in the cage than nestin and suggests local changes in IF composition dependent on the rigidity. In both of vimentin and nestin cases, the size of the cage was

dependent on the rigidity. We could also add that on soft substrate, nestin is even found in a lower amount around the nucleus. Notwithstanding, looking at GFAP perinuclear ring, it was surprising to see higher perinuclear intensity than nestin and vimentin, throughout the rigidity range. The mean intensity around the nucleus in 2 kPa was 3.157, a very similar mean as the 100 kPa condition (3.192), which represent a 0.109% increase otherwise insignificant (Figure 3C). Accumulation around the nucleus of GFAP was generally higher than its IF counterparts, with no rigidity dependency. However, we often observed that on softer substrate, the cage of GFAP often appears incomplete (Figure 3C, bottom panel, black arrow). The cage was generally complete on stiffer substrates, as shown in the top panel of figure 3C.

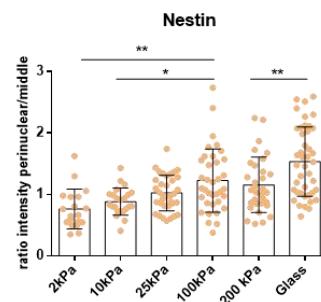
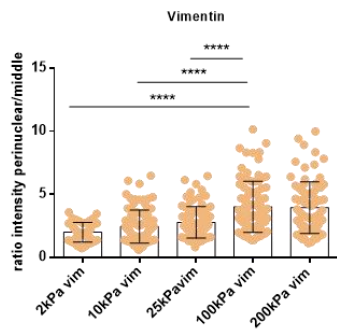
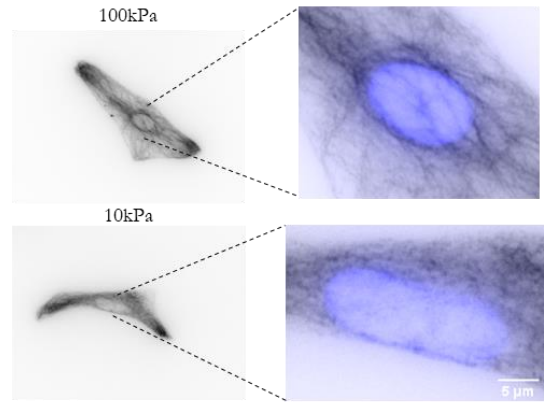
We could also observe a significant increase of GFAP in comparison to nestin and vimentin around the nucleus on 2 kPa: 36.99% increase with vimentin and 75.79% with nestin. On stiffer substrate, here 100kPa, the ratio between the IFs changes. There is no difference between vimentin and GFAP and nestin is significantly lower than both of them (69.34% decrease with vimentin and 61.53% with GFAP) (Figure 3D).

To sum up, vimentin is enriched around the nucleus on stiff substrate in comparison to soft, nestin in a similar way however significantly lower than both GFAP and vimentin and GFAP present a high enrichment around the nucleus throughout the rigidity range.

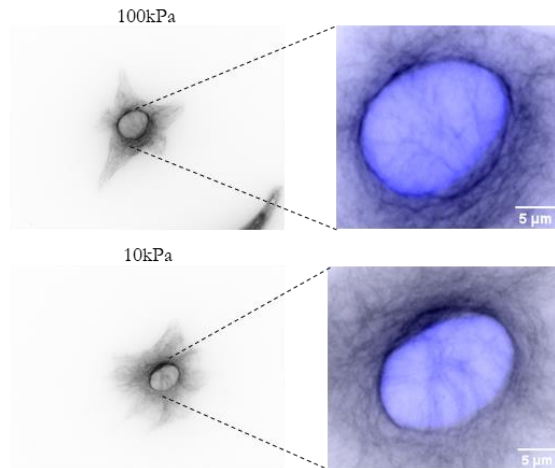
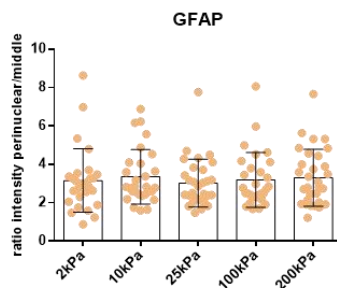
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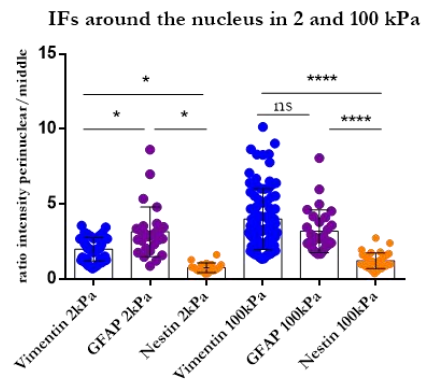
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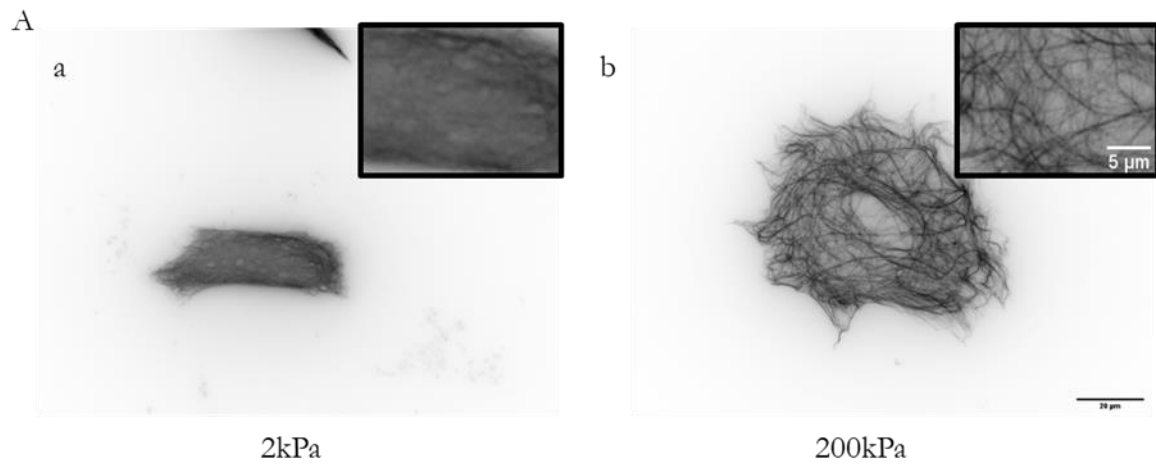


*Figure 3 (previous page) The cage like structure of IFs is dependent on the rigidity. Inverted epifluorescence images of vimentin (A), nestin (B) and GFAP (C) networks of astrocytes plated on 100 and 10kPa accompanied with the respective close-up of the network around the nucleus marked with Hoechst (blue), scale bar= 5µm). The black arrow in (C) points at the incomplete cage. Graphs represent the mean (+/-SD) ratio of perinuclear mean IF fluorescence intensity by the middle of the cell IF fluorescence intensity N=3 scale bars= 5µm. Statistical analyses done using One-way ANOVA followed by Tukey's multiple comparison test (*p<0.05; **p<0.01; ***p<0.0001).*

VII-2-2. Substrate rigidity affects the phosphorylation status of vimentin
Images of the network on different substrate rigidities taken by epifluorescence microscopy showed that higher stiffnesses led to a higher quantity of fully formed filaments than low stiffnesses. On 2 kPa, the network could be described as fuzzy, individual filaments or bundles were less individualised, and clear IF structures were not clear (Figure 4Aa). It led us to believe that IFs are more soluble and/or less bundled on soft substrates. In clear contrast, the network is shown here in figure 4Ab of an astrocyte plated on a stiff 200 kPa substrate, vimentin forms a filamentous network structure.

We hypothesised that the change of network structure on the different rigidity may be linked to the phosphorylation status of IF proteins, since phosphorylation IF proteins can facilitate depolymerisation. To test this hypothesis, we performed western blots, using lysates of astrocytes plated on different rigidities, and using antibodies targeting phosphorylated vimentin at Serine 38. We then normalised against the total vimentin present in the lysate for three independent experiments (Figure 4B). For phosphorylated vimentin in serine 38 (vimpS38), we could find a clear decrease in vimpS38 throughout the rigidity range, going from 4.23 in 2 kPa gels to 1.48 in Glass for control. We tested for the phosphorylation status of vimentin in absence of GFAP, surprisingly, we observed a clear diminution of phosphorylation on 2kPa in comparison to the control (Figure 4B).

To sum up, a decrease of substrate rigidity induces vimentin phosphorylation that results in a high network solubility and less bundled filaments.



B

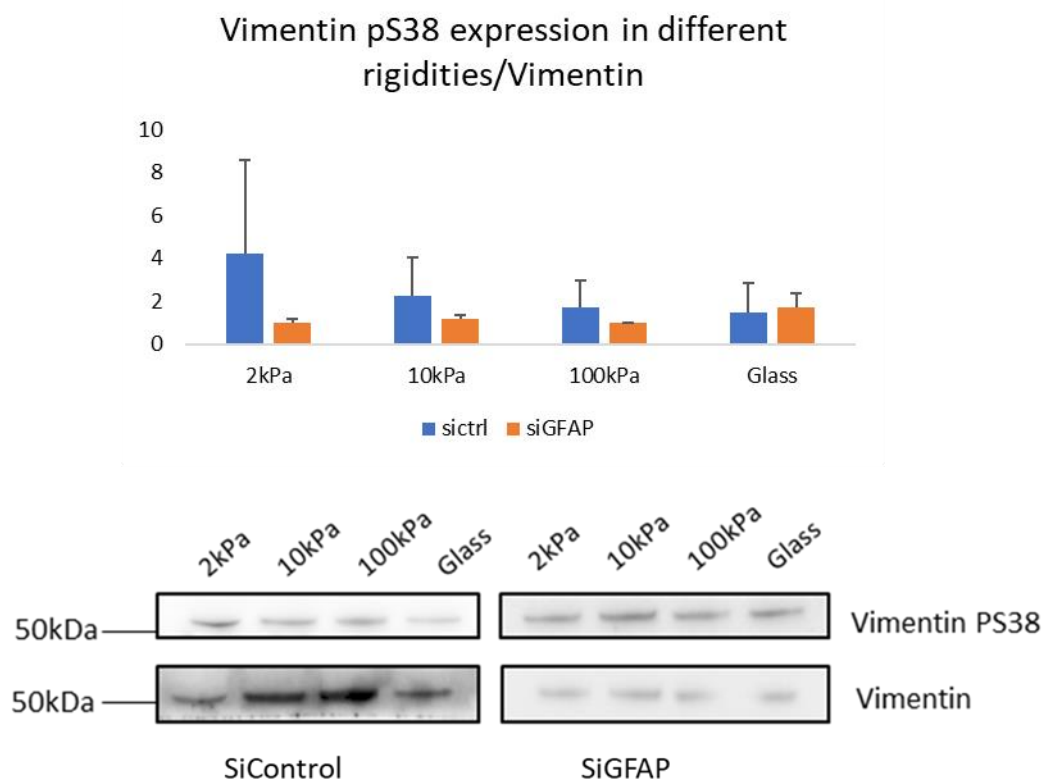


Figure 4 Changes in substrate rigidity affect the solubility of the network. (A) Inverted epifluorescence images of vimentin network of astrocytes plated on 2 kPa (a) and 200 kPa (b) with their respective close up of the network in the middle of the cell scale bar=20 μ m and 5 μ m for zooms. (B) Graphs representing the quantification following western blot ratio between vimentin PS38 and vimentin of lysate of astrocytes plated on polyacrilamide gels of 2, 10 and 100 kPa plus glass, N=3. Representating blot of N=3 experiment.

VII-3. Decreased IF protein expression by siRNA

To study the role of IFs in the effect of substrate rigidity on the nucleus, we designed specific siRNA against the two type III IFs present in astrocyte: vimentin and GFAP (Dupin et al., 2011). We used nucleofection to transfect the siRNA and the cells were left three days before being used. Since nestin and synemin need vimentin or GFAP to copolymerise, we analysed the organisation of the IF network after vimentin and GFAP knock down. To assess the individual roles of the different IF proteins, we also knocked down vimentin and GFAP alone. Staining of the IFs was performed to verify the knockdown. Vimentin, GFAP are barely visible on their respective knockdown (Figure 5Aa-b). It is also the case for the double KD. Nestin however was still present in si2ble conditions but greatly reduced in comparison to the control (figure 5Ac). We could see the collapse of the nestin network and the increase in localisation around the nucleus. Nestin organisation was also different in siVIM and siGFAP: squiggles were found at the extremity of the cell. The siRNAs efficiency was also confirmed by western blot (Figure 5B). By quantifying the expression of IFs normalised by the expression of GAPDH as a loading control, we assessed the percentage of decrease obtained by the KD. For si vimentin, we obtained a 58% decrease in vimentin expression, and 51% for the si double condition. For siGFAP, we obtained a 50% KD and it goes to 89% in si double.

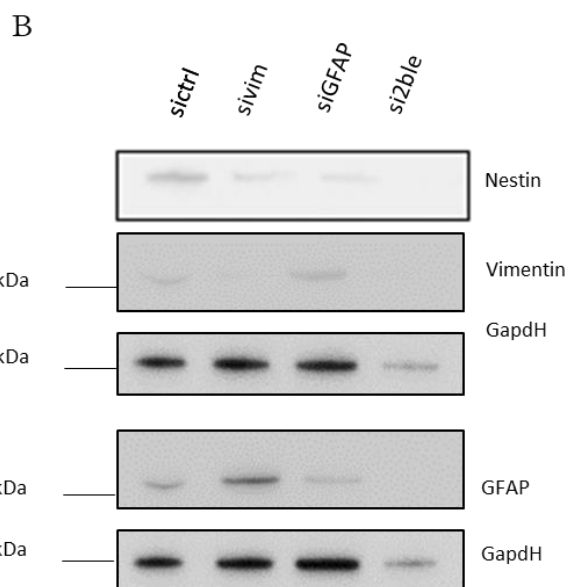
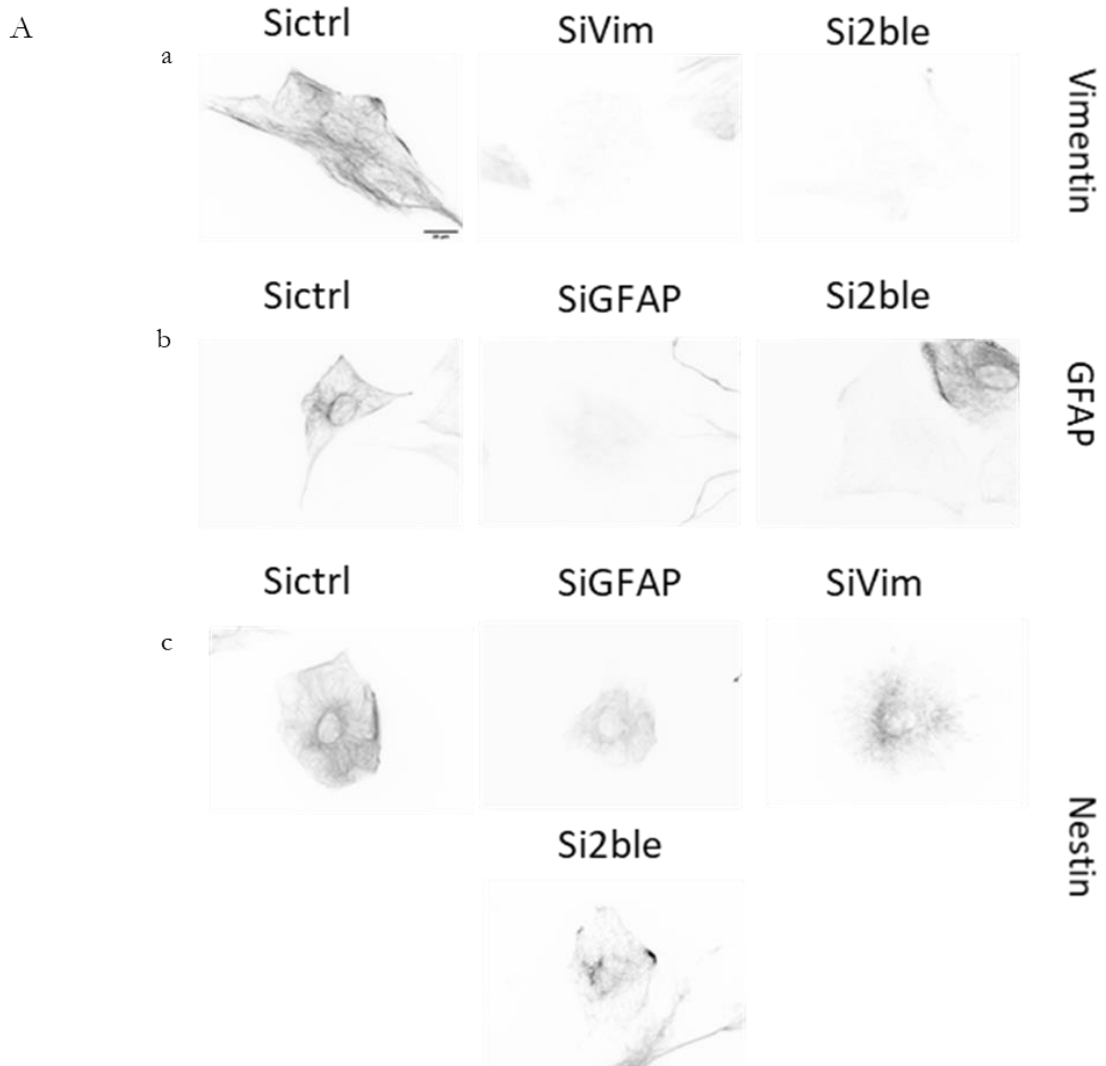


Figure 5 Knockdown of Intermediate filaments. (A) Inverted contrast epifluorescence images of vimentin (a) GFAP (b) and nestin (c) for the different siRNA conditions (sicontrol, sivimentin, siGFAP, siGFAP+vimentin(2ble)). (B) Representative blot of the siRNA condition, showing decrease of IFs. GapdH used as a loading control.

VII-4. IFs mediate some effect of the substrate rigidity on the nucleus.

VII-4-1. Specific IFs affect the size and morphology of the nucleus on soft stiffness

To study the effect of IFs on the response of the nucleus to the rigidity of the substrate, we examined the nuclei of astrocytes plated on different rigidities. The astrocytes were previously transfected with siRNA against vimentin, GFAP or both (see section VII-3-4 above). We used as control siRNA against luciferase. We looked at the 2D area of the nucleus and noticed that as for wild type (Figure 1A), there was a significant increase of area in the control linked to the rigidity (Figure 6Aa). However, in absence of vimentin, this increase was not observed, with all nuclei having a similar size (Figure 6Ab). SiGFAP resembles the control with a significant increase in size between 10 and 100 kPa (Figure 6Ac). Surprisingly, we found that in absence of both IFs, the increase is even more pronounced with smaller nuclei on 2 kPa than the control (95.96 μm^2 on average, against 129.1 μm^2 for the control) and larger on stiff substrates (259.3 μm^2 average against 210.1 for the control) (Figure 6Ad). At 2 kPa, compared to the control, si vimentin the average nucleus area was significantly increased by 29.67%, siGFAP was similar, and si2ble was significantly decreased by 25.65% (Figure 6Ae). Images illustrating these measurements can be found in Figure 6B.

We then quantified the circularity of the nuclei on different rigidities and without IFs. In the control, we found, as expected, an increase in the circularity on glass, but no significant differences between 2, 10 and 100 kPa (Figure 6Ca). A slight significant increase was observed between 10 kPa and the glass under si vimentin condition (Figure 6Cb). This suggests that the increase is more gradual under this condition. However, in the siGFAP condition, there is a significant increase between the 10 and the 100 kPa, as well as between 100 kPa and glass (Figure 6Cc). This difference was probably due to a drop of circularity measurement from 0.4467 in the control to 0.4087 in siGFAP. In the si2ble condition, the trend appeared to be the same as for the control, with a significant increase between 100 kPa and glass, however, with a total decrease in circularity, in all conditions (Figure 6Cd).

VII-4-2. IFs protect the nucleus against nuclear blebbing.

We then assessed the integrity of the nucleus by looking at the number of nuclei that present blebs at different rigidities. Looking at the control, we did not find that the percentage of blebbing nuclei was dependent on the substrate rigidity; however, we found very surprising the overall level of blebs in the control, reaching an average of 39.20% in 2 kPa (Figure 6Da-b). Overall, the number of blebs were significantly increased in the si2ble condition, and on glass, si vimentin (63.87%) and siGFAP (62.62%) also showed a significantly higher number of blebs. This suggests that lack of IFs increases nucleus blebbing independently of substrate rigidity.

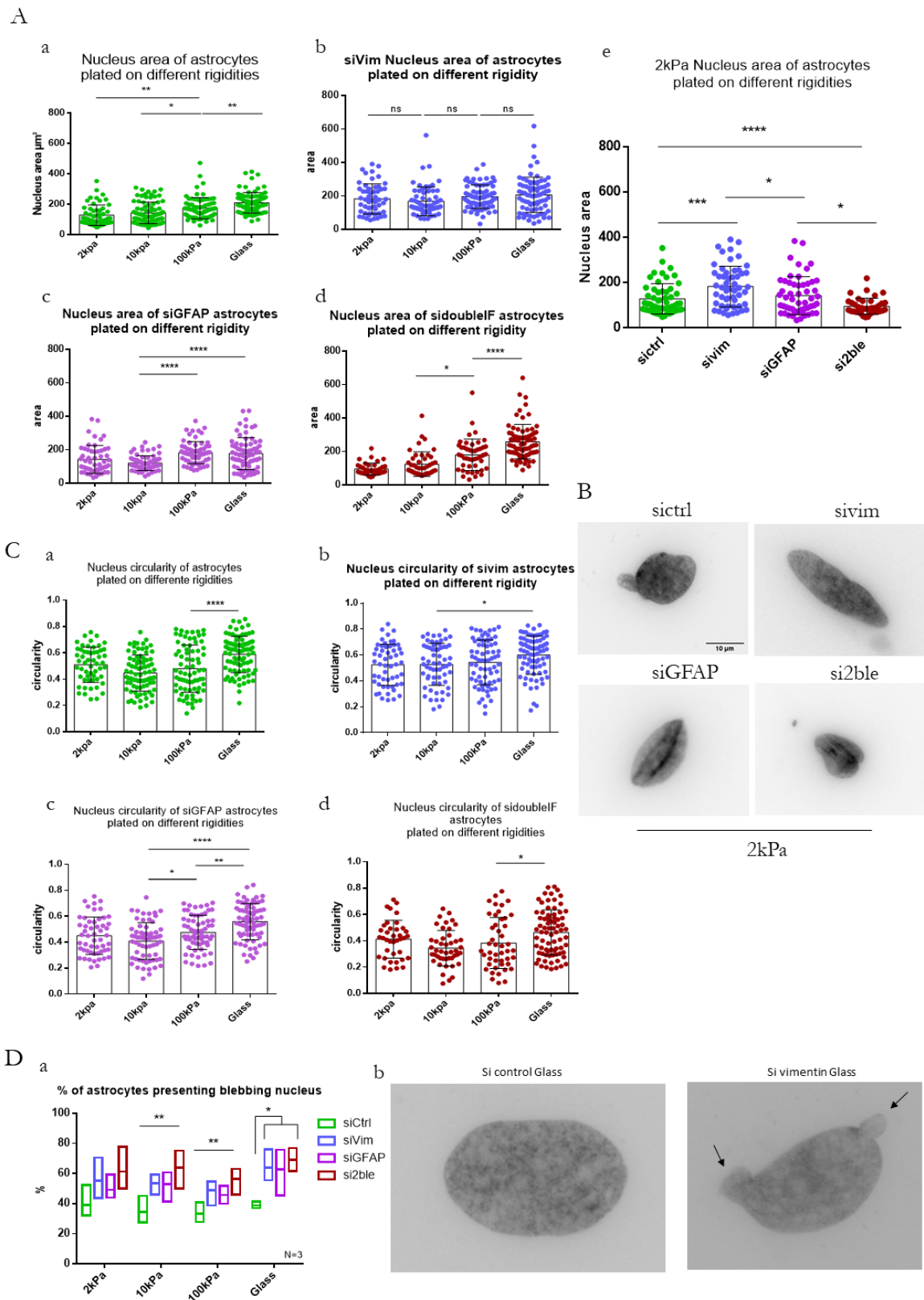
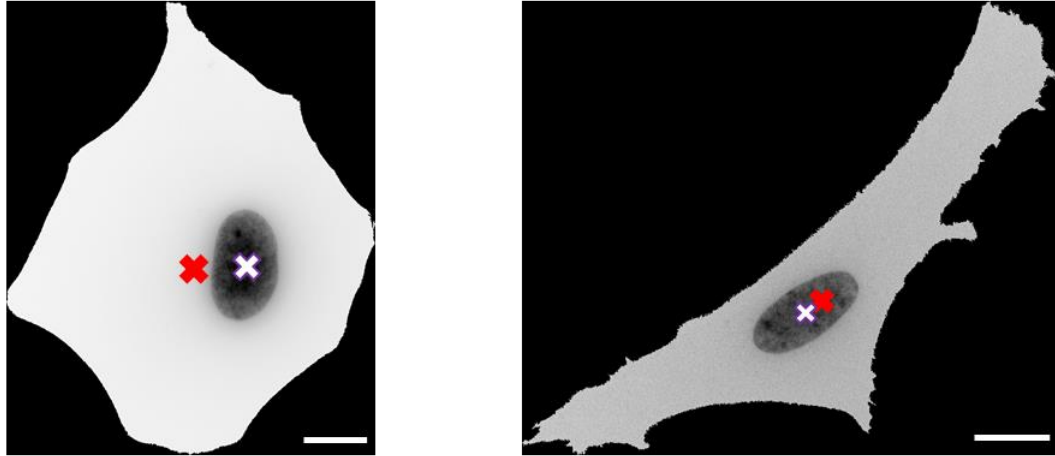


Figure 6 IFs mediate effects of the substrate rigidity on the nucleus. (A) Graphs represent the quantified mean nuclear area (+/- SD) of astrocytes transfected with sictrl (green), siVim (blue), siGFAP (purple) or si2ble (red). Graph A-e represent the combined area of this conditions for 2 kPa. Representative inverted epifluorescence images of nucleus stained with Hoechst are shown in (B) for 2 kPa N=3, scale bar = 10 µm. (C) Graphs representing the circularity score, from the same experiments and conditions as (A). (D) Graph of the percentage of nuclei presenting blebbing in the conditions presented in (A) N=3. Representative inverted epifluorescence images of nucleus of sictrl and siVim conditions. The black arrows are pointed to blebs. Please note that sictrl also present in some case blebs as in (B) top left. Statistical analyses done using One-way ANOVA followed by Tukey's multiple comparison test (* $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$).

VII-4-3. IFs are responsible for the positioning within the cell on different rigidities

To study whether IFs are responsible for the maintaining of the nucleus in a centred position, as seen previously in figure 1C, we calculated the distance between the nucleus centre and the cell centre in the same condition as above: sivimentin, siGFAP and si2ble. We did not see a difference in the conditions of sivimentin and siGFAP compared to the control (Figure 7B top left and middle panels on the and bottom left). However, when both IFs are knocked down, the nucleus appeared significantly less centred compared to 2 kPa and 100 kPa, with an average displacement of 4.261 μm and 10.42 μm respectively (Figure 7B bottom middle panel). It was also surprising to observe that si2ble nuclei seemed more centred in 2 kPa than the control and the two other conditions, and even more surprising that the displacement was increased in 100 kPa compared to the control: 9.759 μm vs 6.519 μm (Figure 7 top and bottom right pannels) as exhibited in the images on the Figure 7A, the nucleus seems more centred on si2ble condition than the control.

A



B

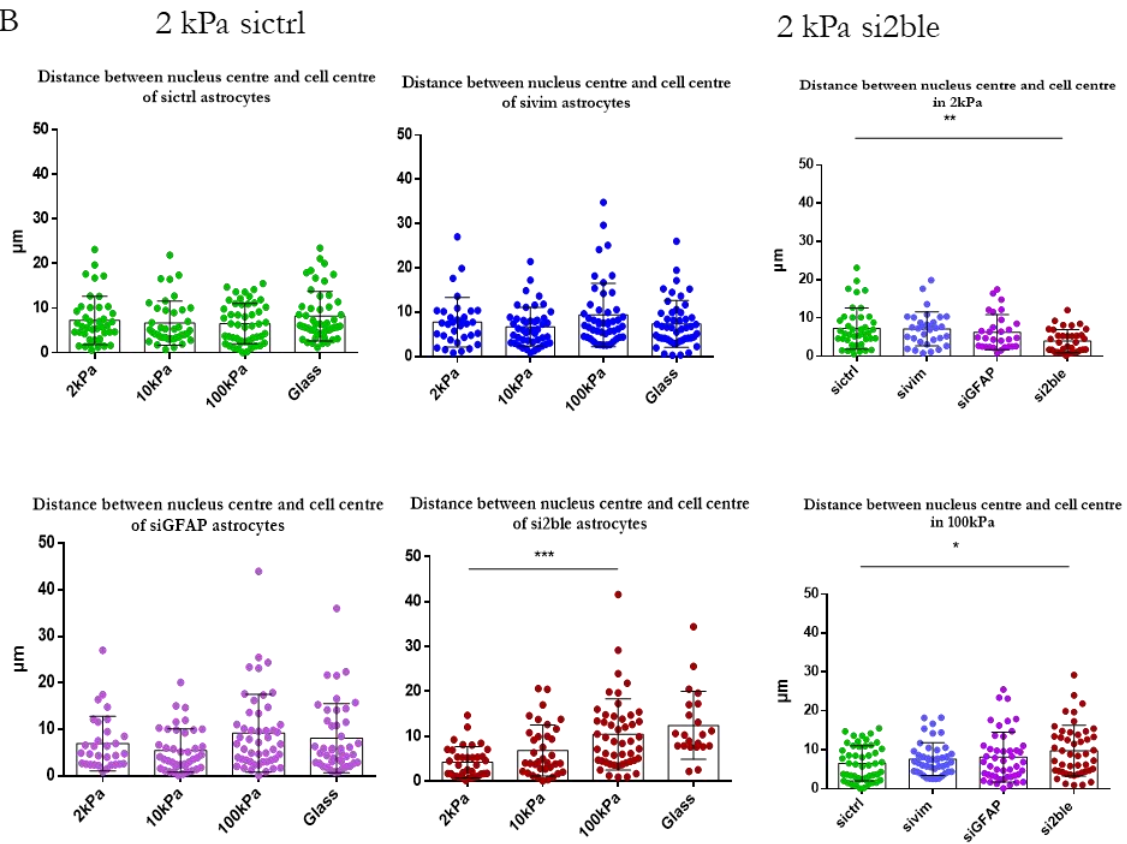
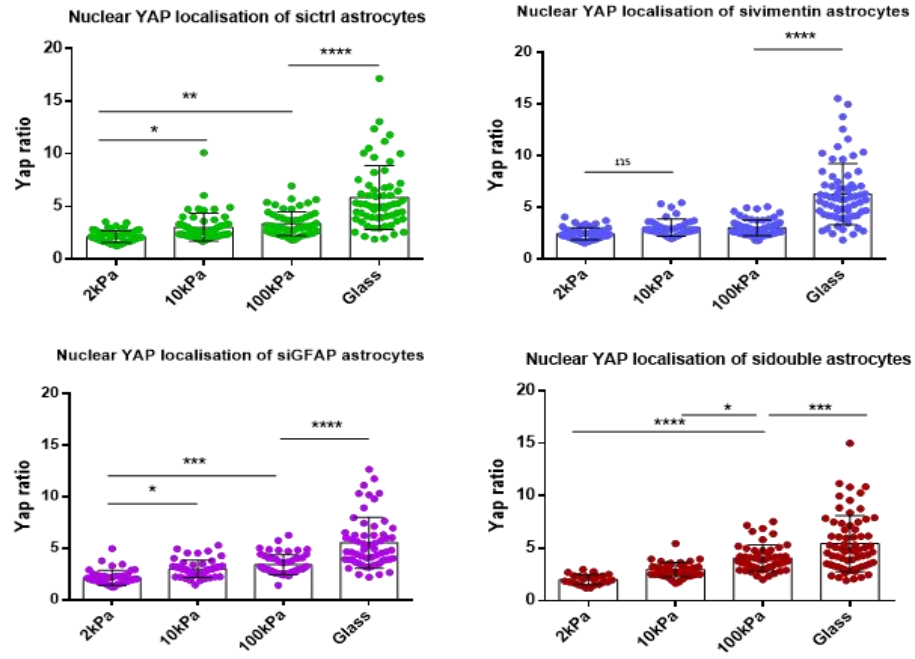


Figure 7 Double depletion of IF affect the positioning of the nucleus. (A) Graphs representing the mean (+/-SD) displacement observed between the centre of the cell and the centre of the nucleus, calculated from coordinates, converted in μm from astrocytes transfected with sictrl (green), sivim (blue), siGFAP (purple) or si2ble (red). The two right graphs represent the combined displacement on these conditions for 2 kPa (top) and 100 kPa (bottom) $N=3$. Statistical analyses were done using One-way ANOVA followed by Tuckey's multiple comparison test (* $p<0.05$; ** $p<0.01$; *** $p=0.0001$).

VII-4-4. Vimentin increases YAP localisation on soft substrate

We then wanted to assess whether the actomyosin network was affected in the KD situation, using the read-out YAP and quantifying its localisation in the nucleus at the different rigidities. We found with the control a similar trend as in the wild type: an increase in nuclear localisation on stiff substrates (Figure 8A). A similar trend is observed in siGFAP: there was a significant increase of YAP levels in the nucleus between 2 kPa and 100 kPa and the increase is even more acute between 100 kPa and glass. However, we only found an increase between 100 kPa and glass in sivimentin cells (Figure 8A). Looking at the images Figure 8B, we could see clearer staining of YAP in the nucleus in 2 kPa than on the control. This was transcribed in quantification, where YAP levels increased significantly by 19.41% in the nucleus in sivimentin cells plated on 2 kPa substrates in comparison to the control. Surprisingly when both IFs are KD, there was once more an increase following rigidities, this time more pronounced, as significance was observed between 2 kPa, and 100 kPa as well as between 10 kPa and 100 kPa (Figure 8B). YAP nuclear localisation is significantly increase on 2 kPa in absence of vimentin.

A



B

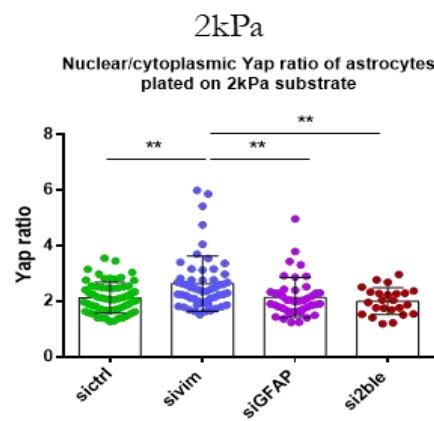
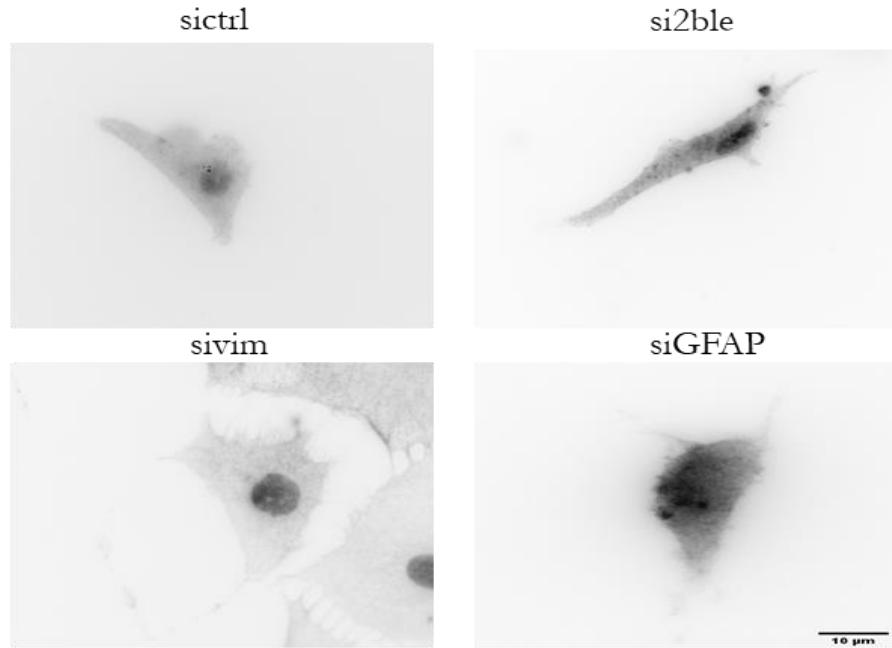
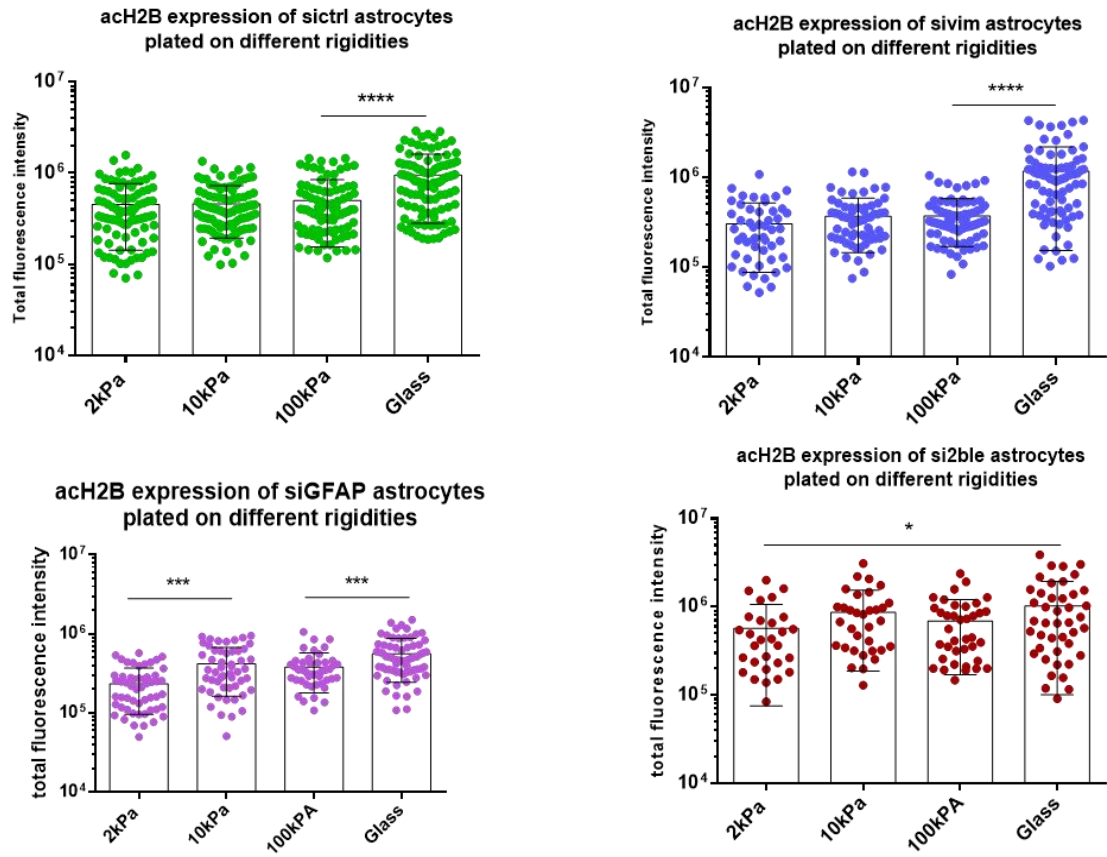


Figure 8 (Previous page) Nuclear YAP localisation is increase in absence of vimentin on soft substrate. (A) Graphs representing the mean (+/-SD) ratio of nuclear Yap against cytoplasmic Yap mean intensity of astrocytes transfected with the same conditions as seen on figure 6, plated on polyacrylamide gels of 2, 10, 100 kPa and glass, N=3. (B) Representative inverted epifluorescence images of Yap in sictrl and sivim conditions. Darker staining represent higher fluorecence intensity. Graph computing the data of the four different conditions on 2 kPa N=3. Statistical analyses done using One-way ANOVA followed by Tuckey's multiple comparison test (* $p < 0.05$; ** $p < 0.01$; *** $p = 0.0001$; **** $p < 0.0001$).

VII-5. IFs affect the rigidity dependent changes in histone PTM.

To study the involvement of IFs on the changes of chromatin conformation observed in WT astrocytes, we stained cells partially depleted of IFs with two different markers of euchromatin: acetyl H2B and acetyl H3, as well as a heterochromatin marker H3K9me3. Graphs are showing the total fluorescence intensity measured on the nucleus. Using a siLuciferase as control, we did not observe a statistical difference between 2 kPa and 10 kPa as seen previously on WT cells for acH2B. We did however find again an increase between 100 kPa and glass (Figure 9A top left pannel). In absence of IFs, no striking differences were observed within the range of rigidity, we did, nonetheless, observe again, a statistically significant increase between 2 kPa and 10 kPa in absence of GFAP and no striking increase was observed between 100 kPa and glass in the double siRNA condition for acH2B. However, at 2kPa, there is a decrease of 32.85% of acH2B expression in absence of vimentin and even steeper decrease of 48.49% in absence of GFAP in comparison to the control (Figure 9B).

A



B

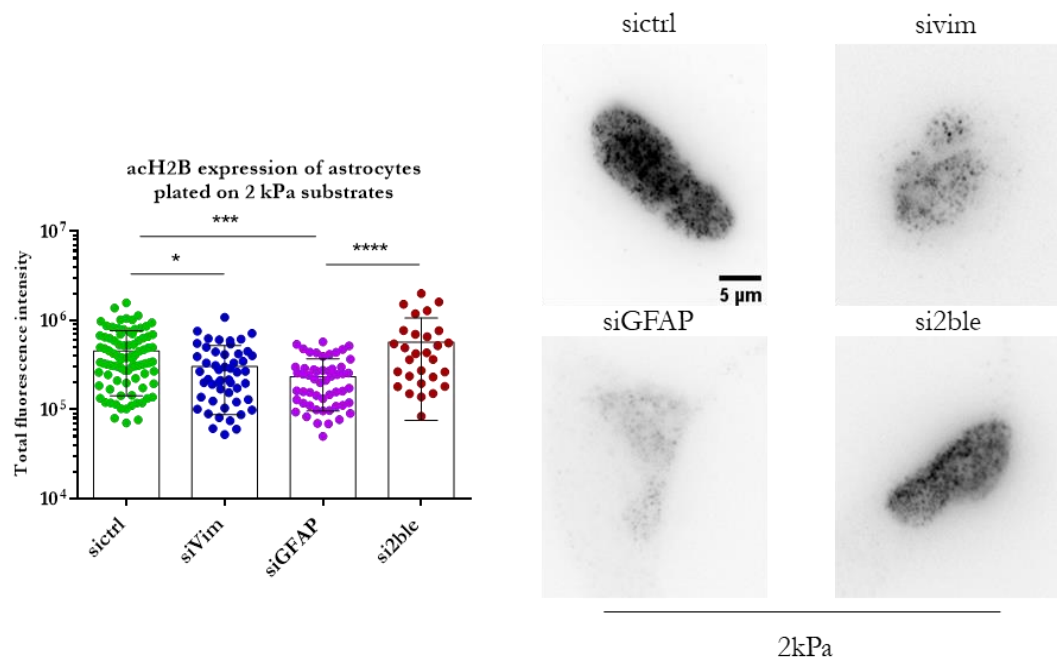


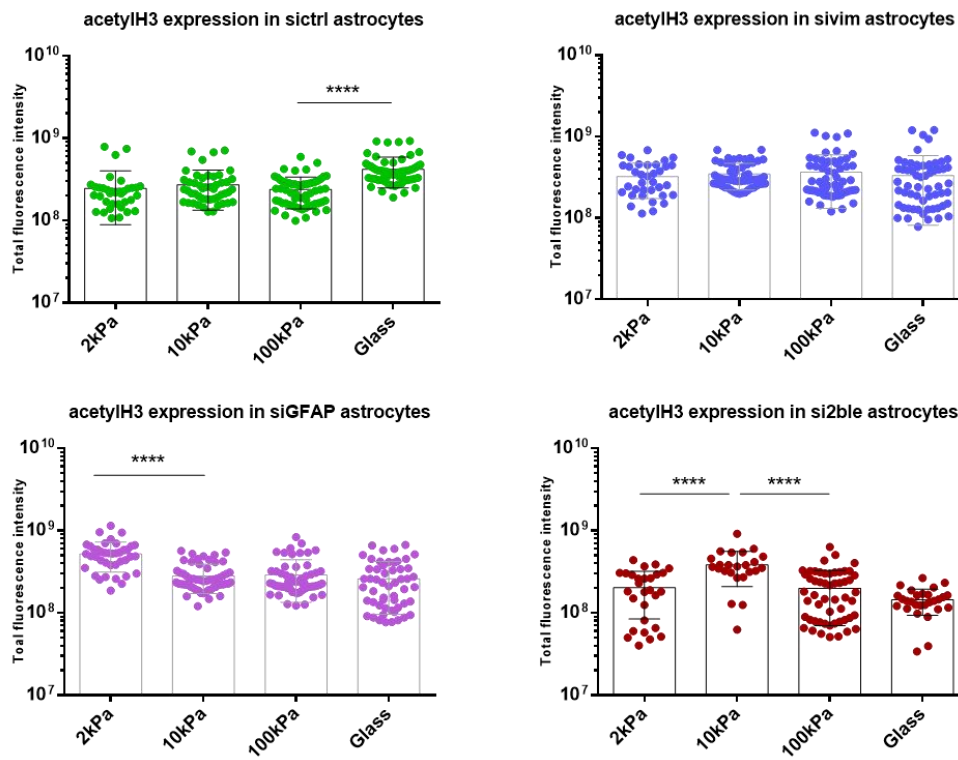
Figure 9 IFs affect acetylation of histone H2B on 2 kPa. (A) Graphs representing the mean (+/-SD) of the total fluorescence intensity of acH2B in the four different siRNA conditions astrocytes plated on 2, 10, 100kPa and glass, N=3. (B) Graphs showing acH2B expression on 2 kPa gels, representative images on the right.

We looked at a second marker of euchromatin: acetyl H3 (Figure 10). A similar trend was observed in the control. However, no significance was found in absence of vimentin throughout the rigidities and more surprisingly, a significant decrease of intensity was found between 2 kPa and 10 kPa in absence of GFAP (Figure 10A bottom left graph). Even more surprising was in the si2ble condition, where a significant increase was observed between 2 kPa and 10 kPa followed by a significant decrease between 10 kPa and 100 kPa (Figure 10A). The decrease was also observed between 100 kPa and glass, however non-significant. Looking at the epifluorescence images, the difference between the four conditions is even more striking on 2 kPa. An increase intensity of 24.56% was quantified between sictl and sivimentin, and an increase of 52.84% of acH3 expression in comparison with the control on 2kPa. However, similar intensities were quantified between the control and si2ble condition (Figure 10B). This was statistically asserted.

Heterochromatin marker H3K9me3 total fluorescence was also quantified on the same conditions (Figure 11). A similar trend can be observed in WT cells. No striking differences were detected in absence of the different IFs. Very similar trends were quantified throughout the conditions: a decrease of intensity between 2 kPa and 10 kPa and an increase between 100 kPa and glass.

To summarise, heterochromatin state doesn't seem to be influenced by IFs, however, the acetylation status of Histone H3 but not H2B depending on the rigidity are affected by the lack of IFs.

A



B

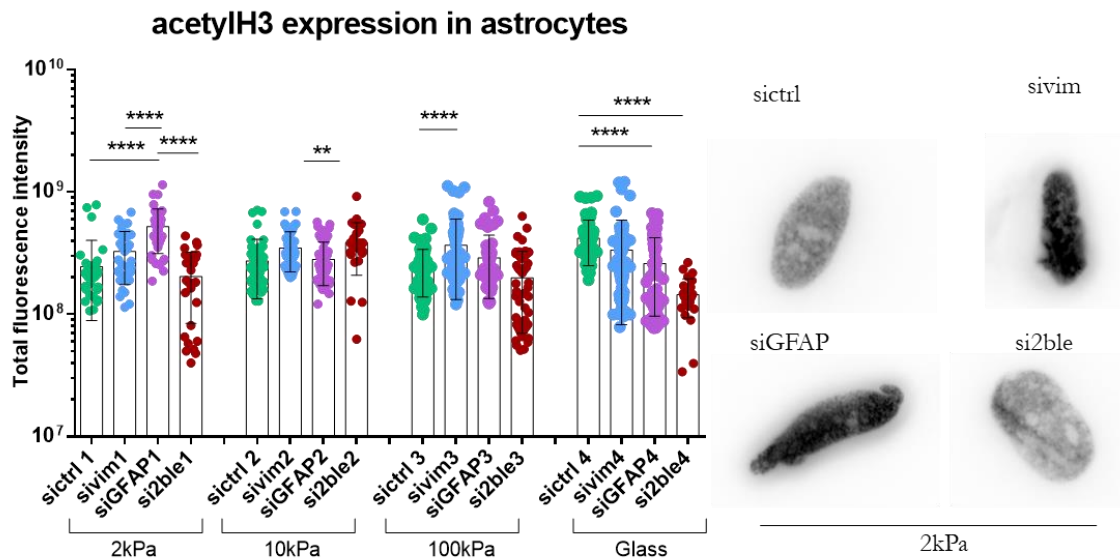
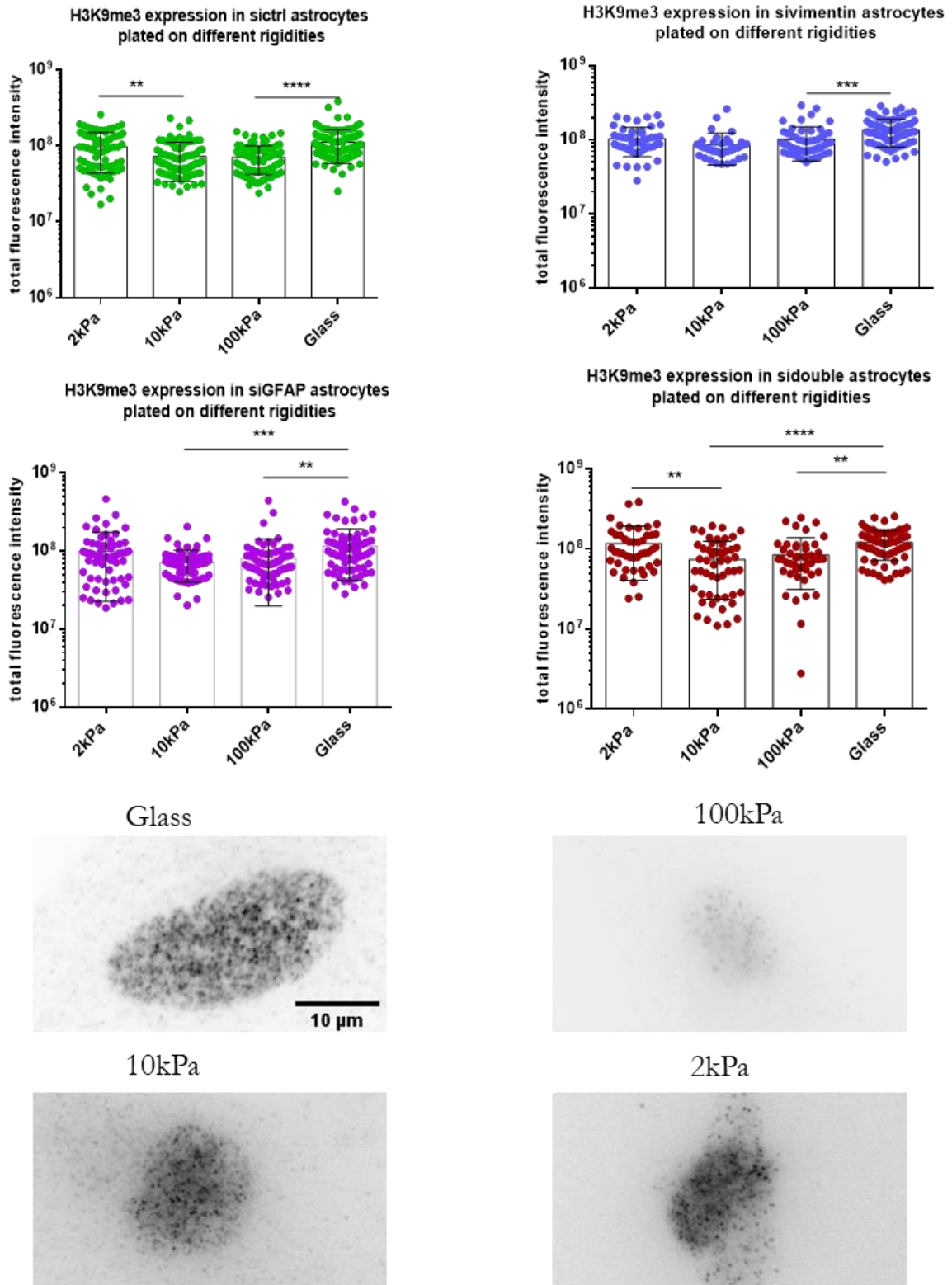


Figure 10 acH3 intensity, changed with rigidity in absence of IFs, in the nucleus. Graphs representing the mean (+/-SD) of the total fluorescence intensity of acH3 (A) in the four different siRNA conditions astrocytes plated on 2, 10, 100 kPa and glass, N=2. (C) The graph is the computation of the graphs (A) categorised by rigidity for better visualisation. Inverted epifluorescence images of nuclei stained with acH3 are representative of the condition 2 kPa. Statistical analyses done using One-way ANOVA followed by Tuckey's multiple comparison test (* $p < 0.05$; ** $p < 0.01$; *** $p = 0.0001$; **** $p < 0.0001$).



Si control

Figure 11 No drastic changes were observed in H3K9me3 intensity in absence of IFs with the rigidity. Graphs representing the mean (+/-SD) of the total fluorescence intensity of H3K9me3 in the four different siRNA conditions astrocytes plated on 2, 10, 100 kPa and glass, N=3. Statistical analyses done using One-way ANOVA followed by Tuckey's multiple comparison test (** $p < 0.01$; *** $p = 0.0001$; **** $p < 0.0001$).

VII-6. Discovery of new interactors of IFs

VII-6-1. Mass spectrometry analyses of potential interactors

Mechanotransduction can happen at the nucleus, at FAs and through the regulation of cytoskeleton components. We therefore looked for the different partners of IFs at the nucleus, FAs and crosslinker of cytoskeleton component through a mass spectrometry screen. To do so, immunoprecipitations of IFs protein coupled with GFP tags were performed in HEK cells, to have a high throughput thanks to their high division rate. The IP was performed with chromotek beads. IFs attached to the beads after being pulled down with their interactors were sent to the mass spectrometry platform of the Institut Curie. We first wanted to assess IF structures on the beads, to understand if potential interactors subsequently found were interactors of monomers or entire filament. Part of the beads was mounted and imaged with epifluorescence. For the example shown in figure 12A, vimentin could be seen forming filaments on the beads, the surface of the beads being “hairy” and fluorescent thanks to the GFP tag. The control GFP alone showed bare and smooth beads. We could now be sure that the interactors found were interactors of fully formed filaments. Filaments were also seen on GFAP and Nestin-GFP beads (data not shown). The IPs were done in triplicate to have quantitative mass spectrometry data. The platform computed a list of potential interactors found in the samples compared to the control, interactors were selected here by their p-values and their enrichment compared to the control. In figure 12B, volcano plots were made with each point representing a protein found in the samples. In x axe represent the fold change, representing the enrichment, the green line represents an enrichment of 2fold. The y axe represents the adjusted p-value, with the red line representing the threshold of significance. The left part of the volcano plot represents the proteins enriched in the control, whereas the right part represents the protein enriched in our samples. Interesting potential interactors are found on the right part, in the quadrant limited by the green and red lines, as well as the column labelled “only in B”, which represents proteins only found in our sample. The clear shape of the volcano proved that the experiment functioned well. From this data, we computed a list of interesting interactors pulled down which each IF protein (Figure 12C). With IF vimentin, we pulled down 4 very interesting targets: firstly HDAC6, (Histone deacetylase 6) was found enriched at a ratio of 2.084 with a p-value of 4.77E-11. This mass spectrometry hit will be studied further in the next section. Vimentin and Nestin were found to both bind with vesicle transport proteins, VAMP7. It is suggested in the literature that IFs are involved in the vesicular transport, it would be interesting to characterise further this interaction. Proteins of the actin regulation pathways were found binding to vimentin, GFAP as well as nestin: RhoA and IQGAP2 and 3. It was very surprising to have a hit for Nesprin-1 in both GFAP and nestin since IFs are known to interact with Nesprin-3 only. The

ratio 1000 on the table means that the protein was only found on the sample and not on the control. Since acetylation of MT is well studied in the lab and that we recently found that it was regulated by substrate rigidity, we wanted to confirm the association with vimentin and the other IFs.

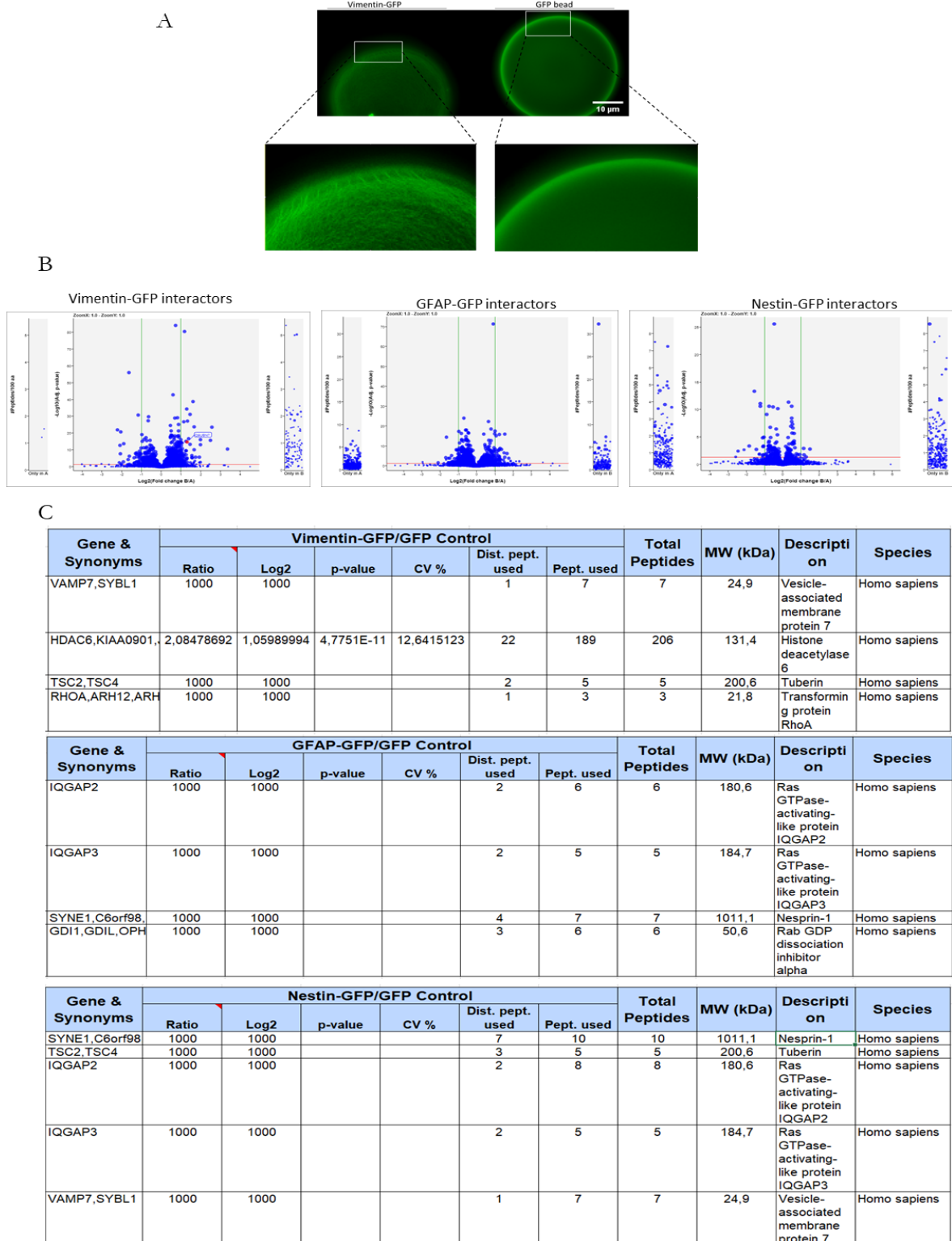


Figure 12 Mass spectrometry identified potential partners of IFs. (A) Epifluorescence images of chromotek® beads after incubation and pull-down with vimentin-GFP (left) and control GFP alone (right), with respective close-ups. (B) Volcano plot of mass spectrometry protein interactor of vimentin (left), GFAP (middle) and Nestin (right). Green lines represent the threshold of 2fold enrichments in either control on the left or sample on the right, the red horizontal line represent the p-value threshold above which bits are significant. Graphs on either side of the volcano represent the bits found exclusively in the control or in the sample N=3. (C) Computed list of interesting potential interactor of each IF proteins.

VII-6-2. Confirmation of interaction between IFs and HDAC6

We found a very interesting target, significantly enriched in our sample: HDAC6. As seen in chapter I of the introduction, HDAC6 deacetylates microtubule. Recently we found that acetylation of MTs is mechanosensitive (Seetharaman et al., 2020), increasing with the rigidity of the substrate. IFs might have a regulation role of HDAC6 indirectly affecting microtubule acetylation in a substrate rigidity manner. Mass spectrometry of IP samples is only giving us a potential interactome of our proteins. After having picked the most interesting target, HDAC6, we needed to confirm its interaction with IFs. To do so, we performed HDAC6-GFP IPs to see if vimentin, GFAP or nestin was pulled down. In figure 13A you can see the IP with the control being GFP transfected astrocytes and our sample HDAC6-GFP condition. We can see that in the input, sampled before pull down, there is more GFAP and Vimentin in the control than in our sample, and an equal amount of nestin. In our IP, we can see a higher amount of vimentin GFAP and nestin in the sample than in the control, suggesting that HDAC6 is pulling down IFs with it. The fact that there was still a small band in the control is in adequacy with HDAC6 being found in the GFP control in the mass spectrometry in a however smaller amount. To verify further the interaction, we performed co-sedimentation assays with polymerised purified vimentin and purified HDAC6 protein (Figure 13C). We mixed the two proteins and centrifuged them. We used as control HDAC6 alone, to be certain that it could not sediment alone and vimentin alone to see if it could sediment, being in a fully formed network structure. In figure 13C, you can appreciate that an equal amount of HDAC6 was loaded in the sample and the control, as well as for vimentin, looking at the input blot. After co-sedimentation, you can see that HDAC6 is found only in the sample of mixed protein and not in both the control. This suggests that HDAC6 sediment with vimentin. This confirmation experiments were performed by Vanessa Roca.

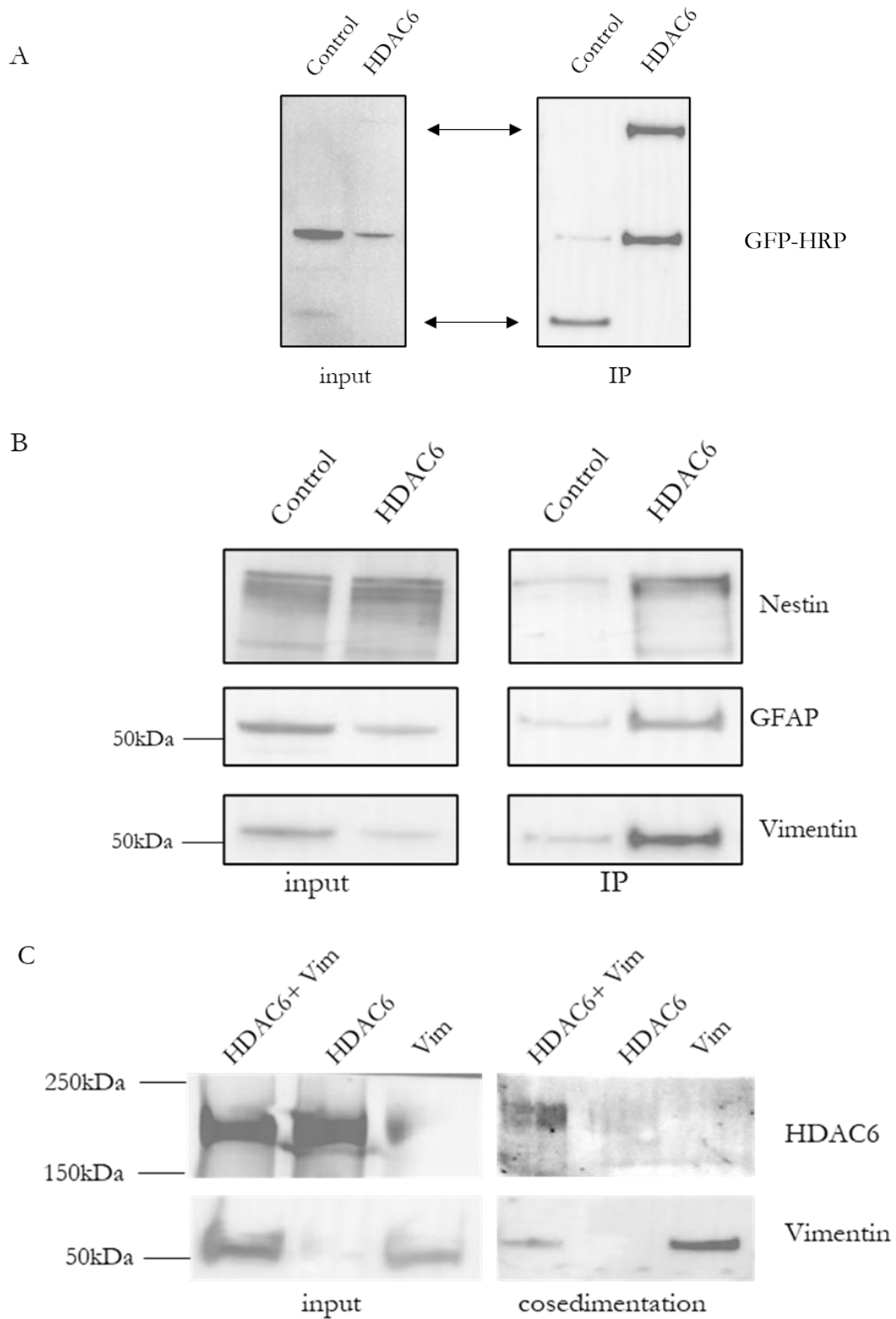


Figure 13 Confirmation of HDAC6 interaction with IFs. (A-B) Immuno precipitation assay were performed using lysates of HDAC6-GFP and GFP control transfected astrocytes. Lysates were incubated with agarose beads for a pre-cleaning and input (left) and IPs (right) were analysed by immunoblotting using GFP-HRP antibody as a loading control (black arrows) (A) and nestin, vimentin and GFAP antibodies. Blot representative of N=3. (B) Cosedimentation of HDAC6 and Vimentin purified proteins were performed. These experiments were performed by Vanessa ROCA.

VII-7. Key messages

In this dissertation we showed that:

- A- Substrate rigidity affects the nucleus area and volume as well as its shape: small and round nucleus is found on soft substrates and flat and large nucleus on stiff.
- B- Astrocytes can sense the difference in tension on different rigidity through the trans localisation of YAP in the nucleus on stiff substrates.
- C- Substrate rigidity changes the acetylation status of histone H2B: There is an increase linked with the rigidity. There is a decrease in trimethylation of histone 3 with rigidity. These changes are linked with structural changes of the chromatin.
- D- The organisation of IFs is changing with the rigidity of the substrate: Vimentin and nestin are forming a denser cage-like structure around the nucleus on stiff substrates and GFAP cage-like structure is always present. This might be linked to the phosphorylation status of vimentin: it is increased on soft substrate.
- E- Lack of vimentin increases nucleus size on 2 kPa and there is more tension visualised with YAP increased nuclear localisation.
- F- IFs are responsible for the maintaining of the nucleus centred position in astrocytes plated on different rigidity.
- G- IFs influence the acetylation of Histone H3 depending on rigidity.
- H- IFs are interacting with HDAC6.

Discussion

VIII. Discussion

VIII-1. Substrate rigidity affects the nucleus.

Polyacrylamide gels enabled us to recreate the different rigidities that cells could encounter *in vivo*. We plated on those gels astrocytes, a model very well described and manipulated in the lab. Primary rat astrocytes have the advantage of possessing a specific combination of IFs that allowed us to compare each IF against the other. We used soft substrates to recreate their physiological environment: stiffness of brain tissue (around 2 kPa). The objective of this part of the study was to detect changes in nucleus physiology when cells experience different environmental rigidities. The mechanism implicated in these changes is particularly important to study, knowing that the physicochemical properties of the ECM are changing in disease. In the case of glioblastoma, an increased stiffness is observed in the tumour environment. Emerging data have shown that the responsiveness of GBM cells to rigidity is an important factor in the promotion of cancer invasiveness. GBM cells that are not responsive to substrate stiffness were described as the most invasive in 3D *in vitro* assays (Grundy et al., 2016). A hallmark of glioblastoma are also changes in nuclear shape and morphology, tightly related to the survival time of patients (Nafe et al., 2003). Understanding how cells and their nucleus change with the rigidity and what molecular actors are at play in a healthy system such as primary rat astrocytes, is therefore crucial.

VIII-1-1. Substrate rigidity affects the size and the shape of the nucleus.

We looked here at how the nucleus morphology, especially its size, shape and position, was changed with the different rigidity. The nucleus is linked to the cytoskeleton through the LINC complex. On hard substrates, a lot of tension is exerted by the actomyosin network on the cell. This creates contractility of the network that affects the nucleus through the link between the LINC complex and the perinuclear cap of actin (Jevtić et al., 2014). Tension on the nucleus can, thus, allow its deformation to enlarge the nucleus. As the soft substrate presents less tension, it led us to believe that the nuclei were smaller in the soft rigidities and increased in size with the rigidity of the substrate. Our findings corroborate this hypothesis, as we found an increase in nuclear area and volume with rigidity (Result figure 1). Looking at the cross section of the nucleus z-stack, a flattening of the nucleus was also observed (Results figure 1A). These results go in alliance with the results obtained in another cellular model: NIH 3T3 fibroblasts (Lovett et al., 2013). They also found a flattening of the nucleus with rigidity, depending on the thickness of the gel. This would suggest that we have the right thickness for our experiment so that the cell does not sense the glass underneath the gel. However, we found that the volume of astrocyte nuclei plated on glass was not significantly changed and remains similar to that of the 2 kPa nuclei. The loss of volume could be explained by the extreme flatness of the nucleus plated on

glass. Recent studies suggest that brain lesions soften the ECM and that they result in astrocyte activation in 3D assays (Y. Hu et al., 2021; Moeendarbary et al., 2017). It would be interesting to look at even softer substrates *in vitro* to see how the nucleus reacts in lesion-like stiffness.

VIII-1-2. Substrate rigidity affect the tension on the nucleus

These changes in the size and shape of the nucleus could be attributed to two different molecular changes. First, direct mechanical tension could play a role. The reduced size of the nucleus on soft substrates could be explained by the reduced internal tensions activated by external rigidity signals. Flattening of the nucleus on stiff substrates could be due to cytoskeletal cables pressing on top of the nucleus (Elosegui-Artola, 2017). Second, nuclear changes could be explained by a change in the structure of the chromatin. An open structure of the chromatin, with unpacked histones, would take more space and might result in enlargement of the nucleus. To test the first hypothesis, we looked at the translocation of the transcription factor YAP into the nucleus. Translocation of YAP into the nucleus is known to be an indicator of tension and is rigidity dependent. YAP is a crucial nuclear relay of mechanical signals (Dupont et al., 2011; Wada et al., 2011). With contractility of the actomyosin network, forces are exerted on the nucleus and it brings the nuclear pores in a more open conformation and letting YAP enter the nucleus (Elosegui-Artola, 2017). It was also demonstrated very recently that YAP might be responsible for the astrocyte response to changes in matrix stiffness. Indeed, inhibition of YAP in primary astrocytes leads to enhanced astrocytic activation on stiff substrates, characterised by an increase in GFAP levels in 3D assays (Y. Hu et al., 2021). Our results match this study, pointing to astrocytes being mechanosensitive, with an increase in nuclear localisation of YAP on stiffer substrates (Results figure 2A). YAP translocation, being sensitive to tension on the nucleus, could mean that changes in nucleus morphology could be attributed to higher tension on the nucleus. This goes along with the circularity of the nucleus on the glass condition being higher. This could mean that an enormously higher tension on the nucleus would make it more circular.

VIII-1-3. Substrate rigidity affect the structure of the chromatin

To test the second hypothesis, we used markers of heterochromatin H3K9me3 and euchromatin acetylH2B. Heterochromatin and euchromatin structures are indicators of gene expression status. In our different conditions (soft substrate and stiff substrate) the differences lie mostly in the tension. In a stiffer environment, there is more tension, more FAs, bigger stress fibres, overall high mechano-regulating proteins. It seems logical to have a higher gene expression in stiffer substrates, which would therefore unpack the chromatin into a less dense structure, take more space and make the nucleus bigger. Our results went along those lines for the euchromatin markers: we observed an increase of expression throughout the range of

rigidity (Results figure 2B). We found a correlating decrease of heterochromatin markers H3K9me3 between 2 kPa and 200 kPa, being especially accentuated between 2 kPa and 25 kPa (Results figure 2C). This shows an increased open structure of the chromatin with rigidity. These results correlate with a recent study on mechanosensitive keratinocytes, where H3K9me3 was found to decrease between 8 kPa and 254 kPa (Laly et al., 2021). The increase in the heterochromatin marker that we observed between 200 kPa and glass could be attributed to the experimentation design; as on the microscope, light needs to go through the gels before absorption and staining could often be observed to be more vibrant on glass than on gels. This also is in line with the steep increase in acH2B with glass. These last results fit well with the ability of chromatin to change nuclear shape (Shimamoto et al., 2017; Stephens et al., 2019). The H3K9me3 marker was very recently found to mediate nuclear stiffness and membrane tension. When the nucleus is deformed by mechanical stretch, the immediate reaction of the cell to counteract the deformation is mediated through calcium-dependent nuclear softening driven by the loss of the heterochromatin marker H3K9me3 (Nava et al., 2020). This fits very well with our observations: high tension on stiff substrate deforms the nucleus by flattening it and making it bigger, which in turn would trigger the loss of heterochromatin structure observed via the decrease of H3K9me3.

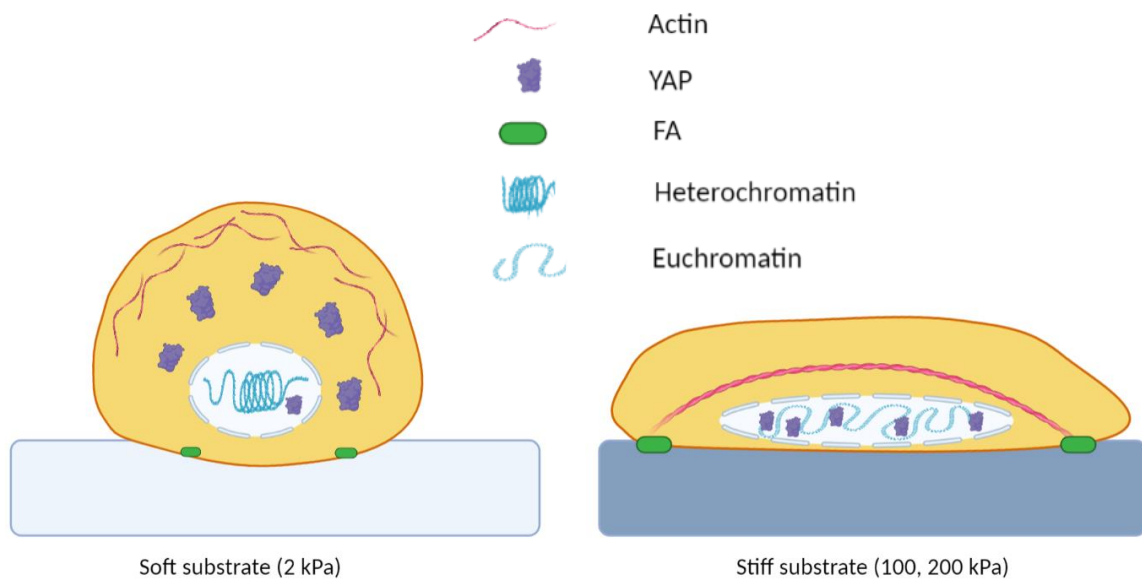


Figure 1 Nucleus morphology on soft and stiff substrates. The nucleus on stiff substrates is flatter and YAP (purple) is found in the nucleoplasm. On soft substrates, the nucleus is rounder and smaller, and YAP is found in the cytoplasm.

Figure 1 represents a schematic recapitulative of our results discussed here combined with what has been seen in the field, in an attempt to create a working model in astrocytes. To push the model further and to validate some of our findings, some additional assays could be performed. To make sure of the structure of the chromatin, assays such as ATAC-seq first described by

Buenrostro and colleagues can show an accessibility map of the chromatin (Buenrostro et al., 2013). The map could be compared between soft and stiff substrates.

VIII-2. The organisation of IFs is changing with rigidity.

VIII-2-1. IFs organisation around the nucleus.

Epifluorescence staining of astrocytic IFs on substrates of different rigidities allowed us to examine the organisation of IFs within the cell. First, we looked at the organisation of the different IFs around the nucleus, to understand and possibly make a link with the morphological changes of the nucleus on different substrate rigidity. We found an increased accumulation of vimentin around the nucleus on stiff substrates in comparison to soft substrates (Results figure 3). It is not surprising to find IFs around the nucleus on stiff substrates, especially glass. IFs, especially vimentin, that forms a cage-like structure have been well documented (Lowery et al., 2015; Patteson, et al., 2019). It is, however, surprising and very interesting to see that the decrease of vimentin and nestin cage observed with the rigidity is in disaccord with previous findings (Results figure 3 A&B) (Murray et al., 2014). In this study, they assert that the vimentin is still forming a cage on 0.2 kPa polyacrylamide gels. Our differences could be attributed to the cell model used. In fact, they used hMSC cells. By comparison of the images of their study, the vimentin network seems to be different from the network in primary rat astrocytes, denser and more linear, almost following actin on stiff substrates. The difference might be due to the species or to the fact that IFs behave differently in every tissue. These cells are originally found in the bone marrow. This could also mean that IFs have a tissue-specific organisation and interaction with the nucleus, at least for the vimentin case. As seen in Chapter I, Section 6.2 of the Introduction, IFs are transported towards the nucleus by a retrograde flow of transverse arc of actin. Stiffer substrates present a higher level of actin stress fibres, and this could mean that a higher retrograde transport of IFs is present in comparison to soft substrates. Ongoing research on the interaction of IFs and the retrograde flow of actin is being carried out by a research engineer in the lab. IFs have long been associated with the nucleus to protect it against mechanical forces, such as migration through confine pores. In those cases, the cage-shaped structure of vimentin protects the nucleus from rupture (Patteson, et al., 2019). It would seem logical that when the cell is subjected to more tension as is the case with stiff substrates, they need higher protection, hence a stronger cage. In this manner, all IFs tested in astrocytes reacted differently. We found a clear increase in the case of vimentin as it was for nestin, however, the latter presented lower intensity overall around the nucleus. It could be explained by the fact that nestin cannot form filaments on their own; thus, the staining is presented dotted, with overall lower intensity. It was however very interesting to note that the GFAP cage around the nucleus was present in all stiffnesses and was even more intense than vimentin (Result figure 3C). GFAP

is very important in reactive gliosis, very recently it was found that a decrease in matrix stiffness increases GFAP levels and therefore activates astrocytes (Y. Hu et al., 2021). This suggests that GFAP is very important in soft substrates and thus, its interaction with the nucleus might be more solicited and this could explain that the cage was observed as strong in soft substrate than stiff substrates. GFAP might not be using the retrograde flow of actin to accumulate around the nucleus, but hijack MT transport (see Chapter I-6-2). To verify this hypothesis, an experiment could be done, plating cells with nocodazole, an inhibitor of MT polymerization and ROCK inhibitors, that are inhibiting the formation of stress fibres and observe if and how the IF cages are forming for vimentin, nestin, and GFAP. In the case of GFAP, it seems that the cage would be specific to astrocytes in the brain, as Schwann cells do not present a cage, it is only present in cancerous schwannoma cells (Utermark et al., 2005). This proves the importance of further understanding the interaction of IFs and the nucleus in a disease setting.

VIII-2-2. Phosphorylation of vimentin makes the network more soluble. Looking at epifluorescence images of IF stained astrocytes, we noticed that clear filamentous structures could not be depicted on soft substrates compared to stiff substrates (Results figure 4). Part of it could be attributed to the experimental design: In inverted epifluorescence microscopy, light had to pass through the polyacrylamide gel. The stiffer the gel, the straighter the light goes to the stained protein. On a soft substrate, the light slightly deviates, making it harder to have an extremely focused image. However, this phenomenon cannot explain all the levels of “fuzziness” observed in the network of IFs on soft substrates. ‘Fuzziness’ could be related to the level of phosphorylation of the network. We looked at the phosphorylation status of vimentin at phosphorylation sites known to be depolymerising vimentin. We found a decrease in vimentin phosphorylation in serine 38 with rigidity, this would mean that at 2 kPa, the network is more soluble (Results figure 4B). Vimentin phosphorylation at serine 38 is mediated by ROCK, preventing vimentin polymerisation *in vitro* (Goto et al., 1998) and depolymerising it in the cell (Eriksson et al., 2004). ROCK is a major downstream effector of small GTPase Rho. It allows the formation of stress fibres. Since there is a diminution of RhoA activity on soft substrates, with lower stress fibres, ROCK may have a more accessible pool of substrate with vimentin, which could explain the increase of phosphorylation. ROCK inhibition decreases phosphorylation and contractility and results in a lower soluble pool of vimentin on a soft substrate. However, inhibition of sole contractility by blebbistatin does not change the soluble pool (Murray et al., 2014). This indicates well the possibility of ROCK phosphorylating vimentin at S38 in soft substrates, making vimentin more soluble. It would be interesting to test this hypothesis by looking at the level of vimentin phosphorylation in cells treated with ROCK inhibitors. A more soluble network of vimentin on soft substrates could also explain the absence

of a clear cage-like structure. Since IFs have a lower amount of filamentous structure, they might not be able to form a lasso structure around the nucleus. From the results about the organisation of IFs around the nucleus in astrocytes, we completed the previous model in figure 2.

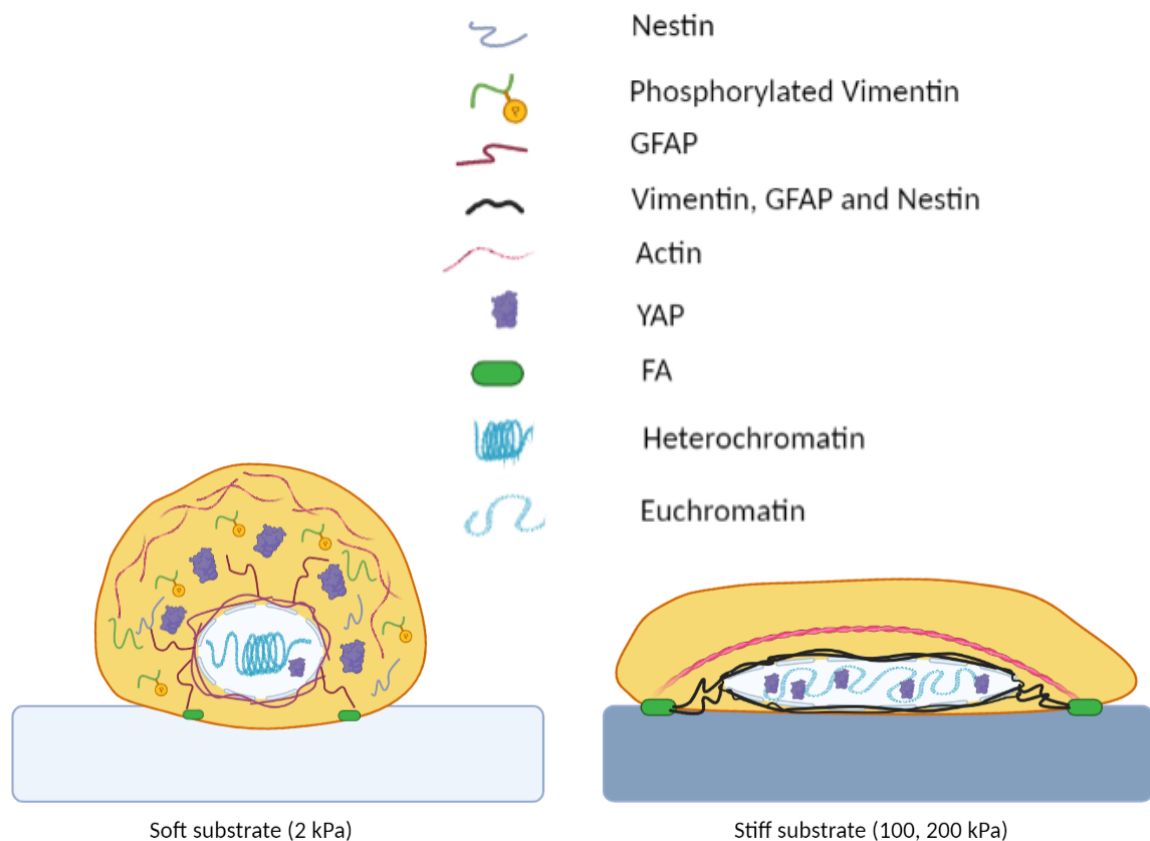


Figure 2 IFs organisation on soft and stiff substrates. On stiff substrates, IFs surround the nucleus in a cage-like structure. On soft substrates, GFAP forms a cage-like structure but not vimentin or nestin and vimentin is found in higher-level phosphorylated.

VIII-3. IFs mediate the effects of substrate rigidity on the nucleus.

We wanted to assess how much IFs intervene in the changes discussed thus far. We know that they have very interesting mechanical properties and are often involved in the states of the disease. Gaining more knowledge about their functions would only be beneficial in understanding how healthy cells can turn into cancerous cells and proliferate. At this scale, we are interested in the direct involvement in the changes mediated by substrate rigidity onto the nucleus. We used siRNA technology to knock down the expression of individual IFs, vimentin and GFAP or both at the same time. This is also one of the limitations of the system: using primary astrocytes, we have to use nucleofection and siRNAs against IFs due to a lack of specific inhibitors. We cannot use Crispr Knock-Out technology on primary cells, so there is still some protein present in the cell. Using a combination of two siRNA doesn't guarantee that both siRNA will be transfected in the same cell. Another limitation is that not all cells are transfected.

This effect is countered by the number of cells analysed being high enough to dilute the effect of non-silenced cells in our conditions.

Knocking down vimentin and GFAP at the same time leads to a decreased level of nestin. This decrease was expected as nestin cannot form homopolymers. It needs to be associated with GFAP or vimentin to form filaments (Bernal & Arranz, 2018). Nestin is still present but is collapsed around the nucleus (Results figure 5). This collapse could be explained by ULFs being transported towards the nucleus by retrograde flow to be incorporated into filaments. However, since there is not enough material to form filaments, the remaining nestin is massed around the nucleus.

VIII-3-1. Lack of IFs changes the morphology of the nucleus depending on substrate rigidity.

We examined the effect of IF KD on the response of the nucleus to changes in rigidity. We first observed that the increase in the size of the nucleus through the rigidity range was not observed in vimentin (Results figure 6A). It was explained by an increase in nuclear area in soft substrates. IFs have been shown to regulate the formation of the actomyosin network. We showed recently that IFs are limiting the coupling of the actomyosin network to the FAs, restricting traction forces. The lack of IFs is responsible for enlarging and reducing FA turnover (De Pascalis et al., 2018). The enlargement of the nucleus in absence of vimentin could potentially be explained by an increase of actomyosin coupling at FAs leading to higher contractility and forces on the nucleus resulting in similar results as the stiff substrates. This hypothesis fits well with our result showing an increase of YAP nuclear localisation in vimentin KD (Results figure 8B). This increase suggests an increase in tension in the nucleus that allows the passage of YAP through the nuclear pores. Lack of GFAP did not provide the same effect and was, in nuclear size and YAP localisation, similar to the control. This would give vimentin a more important role in the tension-dependent effect of substrate rigidity on the nucleus. However, when both proteins are KD, resulting in all IFs KD, surprisingly opposite results are observed: a smaller nucleus on soft substrates in comparison to the control. This could mean that vimentin is working at FAs to control the tension, as it has been shown in several reports (De Pascalis et al., 2018; Gregor et al., 2014) and GFAP is working at the nucleus thanks to its stronger cage-like structure which seem to be contractility-independent, unlike vimentin, to keep its shapes. To complete this study several additional experiments could be performed. First, we do not yet know if the depletion of IFs affects the nuclear volume and flattening linked with the substrate rigidity. Z-stack could be performed using confocal microscopy to understand if the flattening of the actin nucleus by the cable of actin is regulated by IFs.

VIII-3-2. IFs protect the nucleus from blebbing

IFs have been recently shown to act as a protective layer for the nucleus, allowing it to keep its integrity when put under mechanical stress such as migration in confined spaces (Patteson, Vahabikashi, Pogoda, Adam, Goldman, et al., 2019). The cage-like structure around the nucleus protects it against nuclear rupture. We found that the nucleus was presenting increased blebbing, a sign of nuclear rupture when lacking IFs, either individual or all, and thus throughout the rigidity range (Results figure 6D). The gap between KDs and control, however, seemed to be more important in stiff substrates than in soft. This is probably due to less tension and a decreased IF cage-like structure. We note also that the double KD leads in all cases to higher levels of blebbing. All IFs are involved in the protection, since lack of only one leads to higher rupture, there was no compensation by the other ones observed.

VIII-3-3. IFs keep the nucleus centred in the different rigidity

We then looked at the position of the nucleus without IFs. We found earlier that in wild type, substrate rigidity does not affect the nucleus positioning (Results figure 5C). The nucleus is positioned at the centre of the cell as it serves as a control centre when the cell is sparse, nonmigrating, nondividing, and depending on its differentiation status. It is usually centred on astrocytes (Gundersen & Worman, 2013). Nucleus positioning is very important in many cellular processes, and abnormal positioning can lead to disease (Gundersen & Worman, 2013). Several mechanisms are in place to ensure and maintain the position of the nucleus within the cell. The principal factor is the anchoring of the cytoplasmic cytoskeleton at the nucleus through the LINC complex. Logically, the more tension needed, the more anchoring and forces would be needed to keep the nucleus at the same position. We found that with a lack of IFs, the nucleus was more off-centred than in the control on stiff substrates (100 kPa) (Results figure 7). IFs are forming a cage-like structure around the nucleus and interact with the enveloped via nesprin-3 and they attach the cell periphery at FAs. They would be the main suspect when positioning is unpaired. The complete loss of the cage leads to a decrease in the anchorage of the nucleus by the cytoskeleton and could account for the change on position of the nucleus in the stiffer substrate where there is more contractility of the actomyosin network. Since no changes were observed in the conditions of siGFAP and sivimentin, we could say that the loss of one is compensated for by the other for the positioning of the nucleus, and at least one of them is needed to keep the nucleus in a physiologically centred position.

It could be interesting as well to understand if IFs need the LINC complex to act on the nucleus as expected or the confinement of the nucleus alone is sufficient. To test that siRNA against

Nesprin-3 could be used especially. This would retain the link of MT and actin with the nucleus and decoupled IFs. Any changes would be associated with IFs.

Live imaging could be performed on soft and stiff substrates, with and without IFs or knocking down nesprin 3, looking at early adhesion to assess nuclear movement and positioning. This could explain how the positioning is different in singleIFs conditions.

VIII-3-4. Lack of IFs affect the acetylation of histone H3 depending on the substrate rigidity

Knowing that IFs affect the nucleus morphology and position, we further looked at the state of the chromatin structure and gene expression. Acetylation of histone is done at the N-terminal domain of the core histones and allows for a more open structure of chromatin. We looked at the acetylation of the histone H2B first. We did not find obvious changes in acetyl H2B expression in the different siRNA conditions apart from a decrease of expression between siCtrl and siVim and siGFAP condition in 2 kPa (Results figure 9). However, there are several acetylation sites on different histones that could be affected. Next, we look at acetyl histone H3. Acetylation of the histone H3 indicated a remodelling of the chromatin and is triggered by biophysical cues such as elongated nuclei on micropatterns and by stretching, in other words, by applying forces to the nucleus surface, in mesenchymal stem cells (Y. Li et al., 2011). In the control situation, we could observe an increase in H3 acetylation between 100 kPa and the Glass condition (Results figure 10A). Higher mechanical tension would explain the increase in acetyl H3. We then looked at the level of acetylation of H3 without IFs. We observed a change in expression of this marker especially in siGFAP and si2ble condition, where a decrease was observed with the rigidity. Chromatin disassembly, during transcription, is driven by the acetylation of histone H3 (Williams et al., 2008). Levels of H3 acetylation decrease during transcriptional repression and promoter chromatin reassembly. This would indicate that the decrease in acH3 levels observed with rigidity in siGFAP is correlated with a decrease in the open structure of the chromatin and corresponds to a transcription repression phase, induced by the lack of GFAP. When both vimentin and GFAP were reduced, a decrease was also observed, but only between 10 and 100 kPa (Results figure 10A). Since there was no difference with the siVimentin condition, we could assume that GFAP only has a role in the acetylation of H3. Abnormal decreased expression of acetylH3 could lead to cancer genesis, as is the case with hepatocarcinoma (Buurman et al., 2012). We could assess that the decrease was rigidity dependent and that GFAP counteract this effect. Nevertheless, the level of acetylH3 in 2 kPa was significantly higher in siGFAP than in control and siVimentin, and arrive at the same level at 10 kPa and 100 kPa and significantly lower on glass (Results figure 10B). We could speculate

that GFAP limits the open structure of chromatin through acetylation of the histone core H3 on the soft substrate and controls transcription repression on stiff substrates by keeping it open. The difference observed within the two markers acetylH2B and acetylH3 could be explained by the chromatin specificity of the region of chromatin and genes of this acetylation control. IFs and specifically GFAP in astrocytes could control only a subset of genes involved in mechanosensitivity, as acetyl H2B controls the most

commonly active genes NAD pathways (Myers et al., 2003; Parra et al., 2006). It would be interesting to map the gene transcription activity without GFAP to better understand the regulation of genes by IFs on different rigidities. The results of this part are summarised in the table in figure 3.

		<i>siVimentin</i>	<i>siGFAP</i>	<i>Si2ble</i>
<i>Nucleus area</i>	soft	+	=	-
	stiff	=	-	-
<i>Nucleus circ</i>	soft	=	=	-
	stiff	=	-	-
<i>Nucleus position</i>	soft	=	=	-
	stiff	=	=	+
<i>Blebbing</i>	soft	+	+	+
	stiff	+	+	+
<i>YAP nuclear localisation</i>	soft	+	=	=
	stiff	=	=	=
<i>AcH2B</i>	soft	=	=	=
	stiff	=	=	=
<i>AcH3</i>	soft	=	+	-
	stiff	+	=	-
<i>H3K9me3</i>	soft	=	=	=
	stiff	=	=	=

Figure 3 Recapitulative of the results obtained in the nucleus with siRNA against IFs in the rigidities of the substrate. Comparative analysis against the sicontrol: the + corresponds to an increase, = to no changes, and - to a decrease. "Soft" indicates 2kPa polyacrylamide gels and "stiff" 100kPa Polyacrylamide gels.

VIII-4. Geometrical constrain and the nucleus

We used micropatterns to force the cells to adopt a certain shape. The difference with microchannels, for example, is that no external forces are forcing the cell to take a certain shape.

What we observed is that the cell itself rearranged its cytoskeleton network to fit a specific shape, through internal tension and contractility. We designed micropatterns with different widths. The narrower the pattern, the more tension is needed on the nucleus to fit on the pattern. We observed a change in the shape of the nucleus on the different patterns. The aspect ratio of the nucleus decreases with width (Appendix figure iA). This suggests that the nucleus is elongated to fit the lines. Thanks to the confocal images and the reconstitution of the z-stack, actin cables were found on both sides of the nucleus, compressing it (Appendix figure iB). These results correlate with a study using primary endothelial cells, where they found a change in the nucleus shape on similar patterns and using drugs against various cytoskeleton drugs found that depolymerising actin made the nucleus rounder (Versaevel et al., 2012). This goes in line with our observation about actin pushing the nucleus on the side to fit. We demonstrated similar results with an experiment that needs to be completed (Appendix 2). On sticks, stress fiber inhibitor drug ROCK inhibitor leads to a rounder nucleus in astrocytes. To see whether the organisation of patterns changed the IFs organisation in a comparable manner to the study on substrates of different rigidities, we looked at the cage-like structure around the nucleus. Surprisingly, the levels of vimentin around and on top of the nucleus did not change; however, GFAP perinuclear cage was found to increase between stick and rectangle shapes. An important question would be how the cage-like structure of IFs influences the morphology of the nucleus on micropatterns and if it mediates the changes observed.

VIII-5. HDAC6 interaction with IFs.

IFs are present in the cell forming a dense network. They possess, therefore, a myriad of interactors. We attempted to discover new interactors that could fit our model. Using immunoprecipitation of IFs coupled with GFP, followed by mass spectrometry, we found interesting target interactors that were enriched in our IFs-GFP pull-down (Results figure 14). The biggest issue with IPs followed by mass spectrometry is that the protein found in the samples are only potential interactors, as interaction is difficult to assess especially doing a pull-down with filamentous protein. Therefore, it was a challenge and the protocol needed to be optimised. It was indispensable to confirm interaction after finding a target. We sorted the interesting mass spectrometry hits due to their involvement in the cytoskeletal pathway, nucleus localisation, and mitochondria. We set our eyes on a particularly interesting protein called HDAC6. HDAC6 is interesting because its overexpression leads to a collapse of the vimentin network in BJhTERT cells (Rathje et al., 2014). HDAC6 as seen in Chapter 1, has as primary substrate MT and deacetylate it. The acetylation of MTs was recently found in a study by the laboratory to be mechanosensitive. There is a higher acetylation level in stiff substrate compare to soft (Seetharaman et al., 2020). We wondered if IFs were implicated in the mechanosensitive

response of MT acetylation to substrate rigidity, through its potential interaction with HDAC6. However, we first needed to confirm the interaction of IFs with HDAC6. We performed reverse IPs, transfecting astrocytes with HDAC6-GFP and looking for IFs to go down with them. We found IFs coprecipitating with HDAC6-GFP. The limitation of this assay is that HDAC6 is known to bind to MTs and actin, IFs are also known to bind to MTs. Therefore, it is difficult to know if the interaction is direct or indirect. We are currently optimising this reverse IP using drugs depolymerising MT and actin to eliminate their interaction with HDAC6. We also performed cell-free co-sedimentation essays showing purified HDAC6 sedimenting with polymerised purified vimentin, confirming interaction. The next step would be to define the expression of HDAC6 on substrates of different rigidity in the presence and absence of IF. We started by staining HDAC6 on substrates of different rigidity and quantified the total level of HDAC6. We found a decrease between 2 and 10 kPa for the control, while a significant increase was found in sivimentin (see Annex 3). In 2 kPa, the level of HDAC6 is significantly higher in the control than for sivimentin, siGFAP and si2ble conditions. This result suggests that HDAC6 is mechanosensitive and IFs could be involved in its mechanism. To carry on the characterisation of the interaction between HDAC6 and IFs, a mapping of their interaction could be performed by doing IPs with fragments of IFs (tail-less, rod, head-less proteins). We could also look at the organisation of IFs with the inhibition of HDAC6 using tubacin. And finally looking at the colocalization of IFs and HDAC6 on substrates of different rigidities using epifluorescence microscopy.

If IFs effect of the substrate rigidity is mediated by HDAC, inhibition of HDAC6 via well-established drug tubacin could be an interesting target for therapy against GBM progression. It is therefore crucial to understand better the interaction between HDAC6 and IFs.

IX. Perspective

This study is just the start of discovering the role of IFs in the mechanotransduction at the nucleus. Further study would be needed to advance the understanding and intrinsic mechanism linking nucleus, IFs and ECM. Our study points towards a role of IF in the nuclear response to external mechanical stimuli, but the exact mechanism by which it occurs still needs to be deciphered.

We could show that the interaction of IFs with the nucleus depends on the rigidity. We still need to know how the cage is forming, and what effect of a rupture of the cage have on the nucleus. For this, a FRAP experiment targeting the specific zone of the perinuclear cage in astrocytes transfected with IFs-GFP could be done to assess the recruitment of the new IF ULF into the perinuclear space and to assess any changes in the nucleus when disrupting the cage and compare the changes with cells plated on soft substrate where the cage is not observed. Decoupling IFs from FA, we could look at the organisation change of IFs around the nucleus. If the cage is not present anymore, it would suggest that its formation is promoted by the association of IFs at FA, making the link between FAs and the nucleus.

Since we found a difference in the chromatin structure on different rigidity and absence of IFs, we could use a technique called ATAC-seq to map the chromatin accessibility, comparing it with the different solutions. Mass spectrometry quantitative analyses of cell plated on soft and stiff substrates could be very interesting but rendered difficult but the small sample of available cellular material given per gels. This could be more specific than RNA-sequencing. We could quantify differential protein expression between stiff and soft substrates. Using cells depleted of IFs, we could find which proteins are mecano-regulated by IFs.

Since IFs are overexpressed in glioblastoma, we could look at the effect of overexpression of IFs in astrocytes, using specific targeted genes to see if we reach a more similar phenotype.

Using specific drugs inhibiting the partner of IFs discovered with mass spectrometry, such as tubacin which inhibit HDAC6 expression, we could determine their regulatory effect on IF network.

Clinical application:

Since changes in ECM properties is observed in glioblastomas, unresponsiveness to the changes promote progression, assessment of the nuclear changes could indicate the cancer aggressivity and characterisation of the accumulation of IFs around the nucleus could be used as a marker for cell responsiveness to the substrate.

Astrocytes having specific combination of IFs, it would be interesting to further understand the importance of this combination in the cell. The reexpression of vimentin and nestin in astrogliosis and in most glioblastoma lines, assessing the change in the balance of IF, overexpression of GFAP, and the reduction of vimentin and nestin in these cells can influence the responsiveness of GBM cells to the substrate, with GFAP more enriched around the nucleus. This could lead to a reverse phenotype and lower aggressivity.

Appendix

Appendix 1

Geometrical constraint affects the nucleus of astrocytes

To study the effect of geometrical constraints on astrocytes, we designed a micropatterning mask with specific shapes that are increasingly narrow, forcing the cell to spread in a constraint space. The different patterns help to reproduce the shape that cells need to adapt when they squeeze through ECM pores. It ultimately helps to understand the impact of the constraint on the cell and how the cells modulate the morphology of their nucleus. We designed infinite lines with widths of 5, 10, and 25 μm , as well as square and rectangles having different widths but the same area of 2500 μm^2 , previously found to be the most adequate area for astrocytes to spread correctly (Seetharaman et al., 2020).

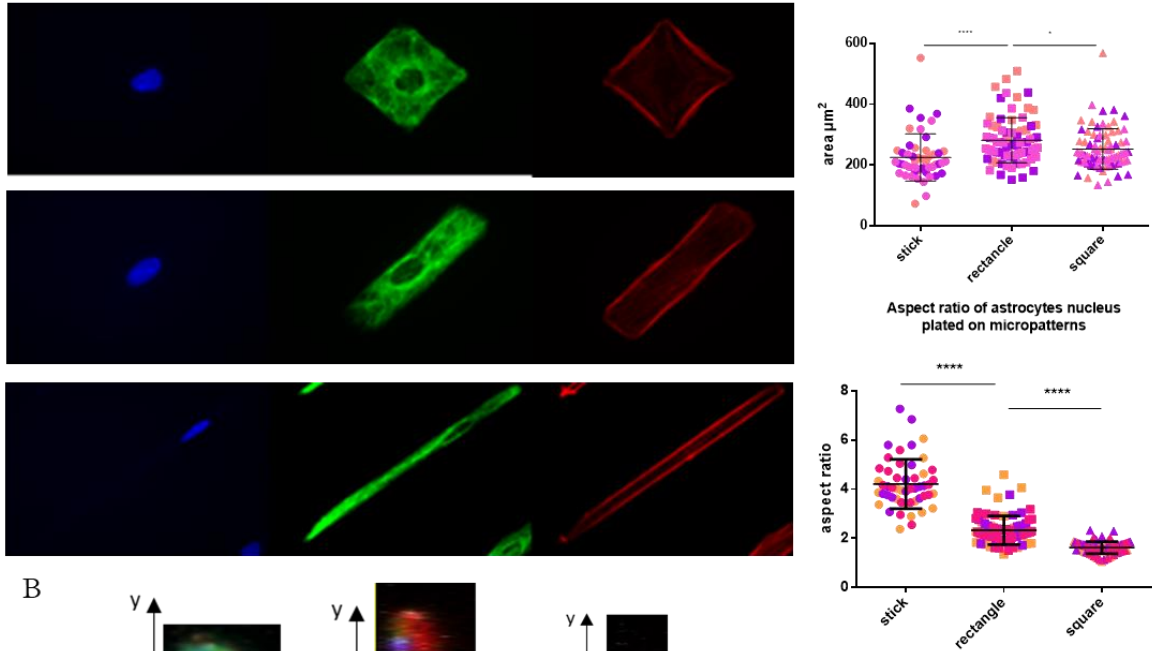
Geometrical constrain affect the size and the aspect ratio of the nucleus

After dapi staining, we found that the nucleus area increased significantly between the stick shape (10x250 μm) and the rectangle (25x100 μm) and then decreased significantly between the rectangle and the square (50x50 μm) (Figure iA). This suggests that constraining the cell leads to an increase in the nuclear 2D area. We looked closer at the shape of the nucleus, especially how elongated it was, to see if the cell was able to change the shape of its nucleus to fit the pattern. Using the measurement of the aspect ratio, we found a clear and significant decrease between the stick (AR~4.1) and the rectangle (AR~2.2) and between the rectangle and the square shapes (AR~1.8) (Figure i). This means that the nucleus is adapting a more elongated shape to narrow patterns. Looking at the Z sections of the patterns, we found that the nuclear volume seemed to increase on wider lines, going from a mean of 446.194 μm^3 for the 5 μm line to 480.506 μm^3 for the 10 μm , to 644.036 μm^3 for the 25 μm line. This would correlate with the increase in volume observed with the substrate rigidity: flatter nucleus with higher volume. However, only two experiments were done with 3 cells per condition. Therefore, to verify these results, more nucleus volumes need to be calculated on the different patterns. So far what we could see is that the nucleus in the smaller patterns was rounder, with the actin cable pushing on the side and the vimentin surrounding the nucleus. On wider patterns, the nucleus was completely flattened and actin and vimentin were found mainly on the side (Figure iB).

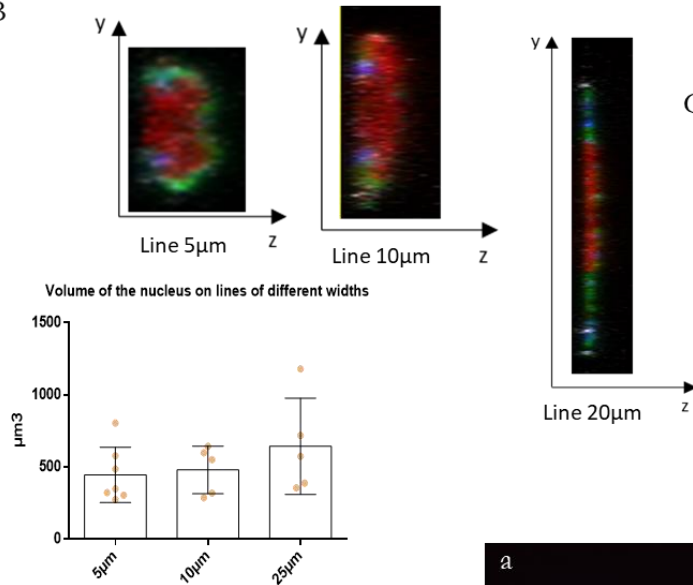
Geometrical constrain affect the nucleus morphology, size and position and integrity

We then quantified the percentage of nuclei going out of the patterns on infinite lines of different widths on four different experiments. We found that there is an average of 32% of cells with a nucleus that exits the pattern on 5 μ m width lines, this percentage drops to 12.67%

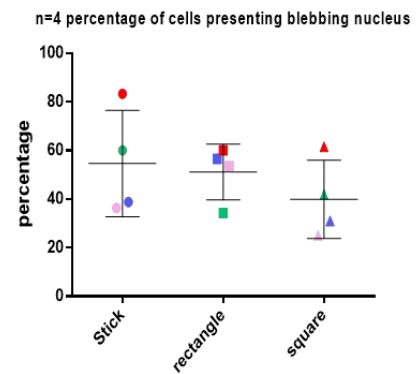
A



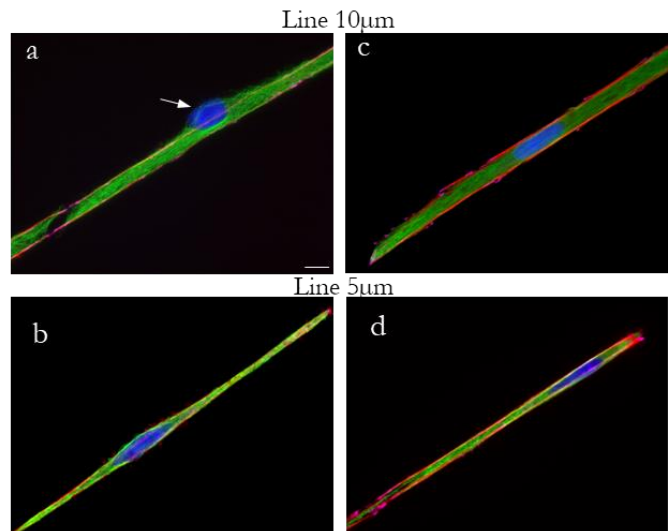
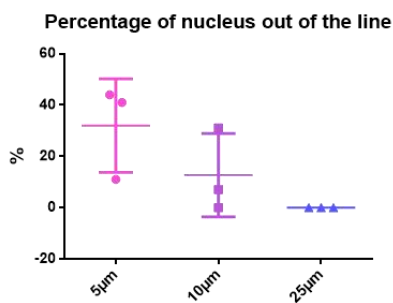
B



C



D



*Figure i (previous page) Geometry constrain affect the nucleus morphology. (A) Epifluorescence images of WT astrocytes plated on square, rectangle and stick-shaped micropatterns of $2500\mu\text{m}^2$, stained for Dapi (blue), Vimentin (green) and actin (red) scale bar= $10\mu\text{m}$. Graphs represent the mean (\pm SD) area of the nucleus (top) in μm^2 and the mean aspect ratio (bottom) on the different micropatterned shapes $N=3$. (B) Confocal z -stack orthogonal view of the astrocytes structure on infinte micropatterned line of 5, 10 and $25\mu\text{m}$ widths stained with dapi (red), vimentin (green) and actin (blue). Graph represent the mean (\pm SD) volume of the nucleus delimited by dapi, from confocal stack $N=2$, $n<5$ / conditions. (C) Graph representing the percentage of nucleus presenting blebs on the different micropatterned shapes. (D) Graph represent the percentage of cells presenting a nucleus going out of the shape of the pattern, as shown by the white arrow on the representative images of 10 and $5\mu\text{m}$ lines. Cells were stained for dapi (blue), vimentin (green) and actin (red). Statistical analyses done using One-way ANOVA followed by Tuckey's multiple comparison test ($*p<0.05$; $***p<0.0001$).*

on $10\mu\text{m}$ line and down to 0% on $25\mu\text{m}$ (Figure iC). You can appreciate in figure iC in panels a and b, the nucleus going out of the pattern. Panels' c and d show the nucleus being compressed and staining within the line. The nucleus could go out of the pattern on either side (b) or only on one side (a).

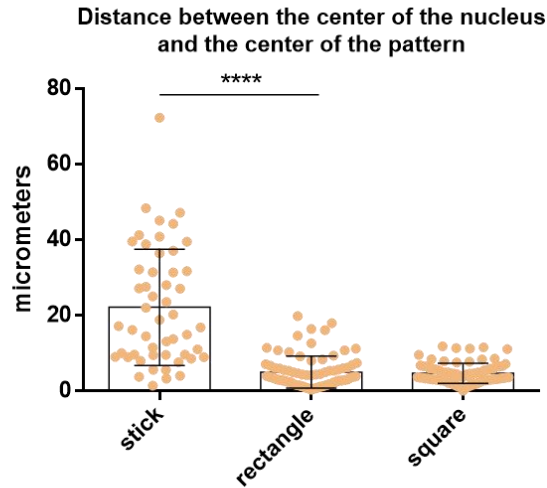
We then assessed the integrity of the nucleus on the different patterns (stick, rectangle, and square). To do so, we quantified the number of nuclei that presented blebs. Blebs are characteristic of a leakage of the nucleus. 54.63% of the nuclei on sticks presented blebs. This number slightly drops to 51.09% on rectangles and drops a bit more on squares to arrive at 39.86%. As for the substrate rigidity, the nucleus seemed to be blebbing in all tension conditions. The increase of nucleus rupture on narrower patterns can, however, not be certain as this increase was not statistically significant.

Positioning of the nucleus within the patterns was the next step we wanted to assess. As for the rigidity, we calculated the distance between the centre of the pattern and the centre of the nucleus. In rectangles and squares, the nucleus was approximately centred, with an average displacement of $5.059\mu\text{m}$ displacement for rectangles and $4.746\mu\text{m}$ for square. The displacement is jumping to an average of $22.21\mu\text{m}$ on sticks. Nuclei were found throughout the form. Some were at the back, some centred, one was even $72\mu\text{m}$ away from the centre (Figure iiA).

On the images presented in Figure iB, we observed IFs enveloping the nucleus on narrower patterns. To characterise this potential accumulation of IFs around the nucleus, we quantified the fluorescence of vimentin and GFAP on top and in a $2\mu\text{m}$ ring around the nucleus. In comparison, we also looked at actin around the nucleus. Surprisingly, the quantification for vimentin did not show any differences between stick, rectangle and square (Figure iiBa). On the other hand, a significant decrease in actin around the nucleus was observed between stick, rectangle, and square. For GFAP, we found a significant increase between stick and rectangle,

plateauing for square (Figure iiBb). GFAP but not vimentin appears to be accumulating around the nucleus in wider patterns.

A



B

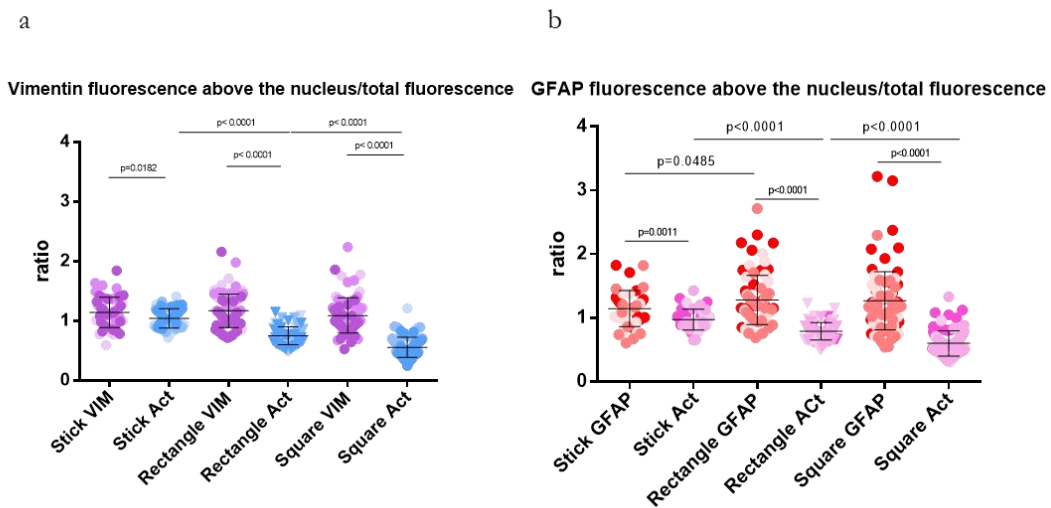


Figure ii IFs and actin organisation was changing with geometrical constrain. (A) Graphs represent the mean (+/-SD) ratio of vimentin (left) and GFAP (right) and corresponding actin (act) fluorescence on top and within a $2\mu\text{m}$ ring around the nucleus of WT astrocytes micropatterned with different widths. $N=3$. (B) Mean (+/-SD) displacement measured between the cell centre and the nucleus centre. Statistical analyses done using student *t*-test (A) and One-way ANOVA followed by Tuckey's multiple comparison test (**** $p<0.0001$) (B).

AR nucleus of astrocytes plated on micropatterns

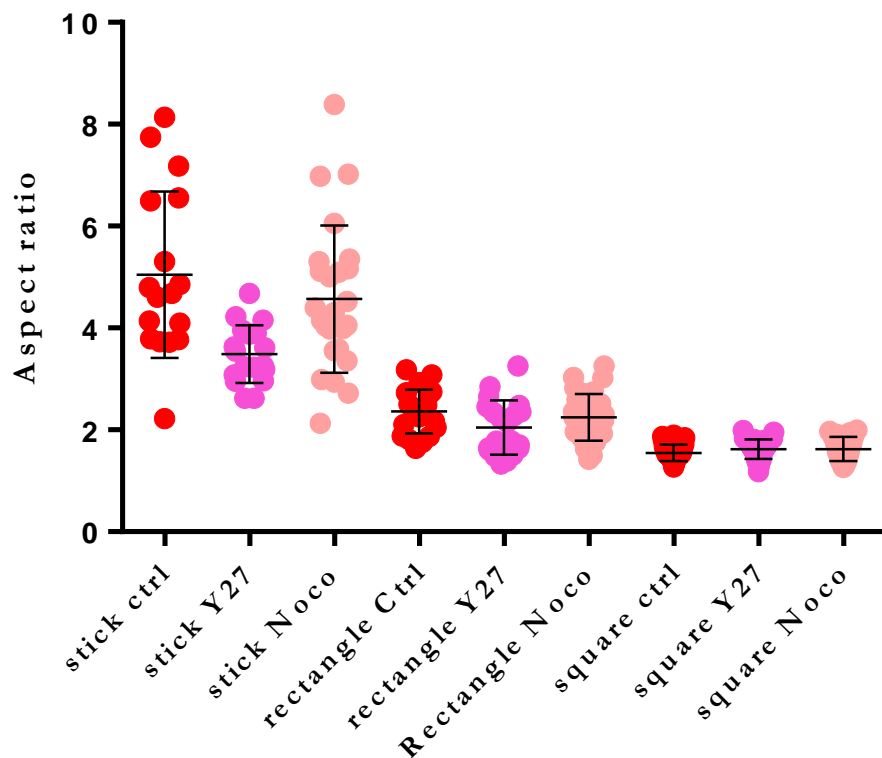


Figure 1 Aspect ratio of astrocytes plated on micropatterns of different widths. Cells were treated for 2 hours with ROCK inhibitor Y27 and nocodazole to inhibit MT polymerisation and then fixed and stained for DAPI.

Appendix 3

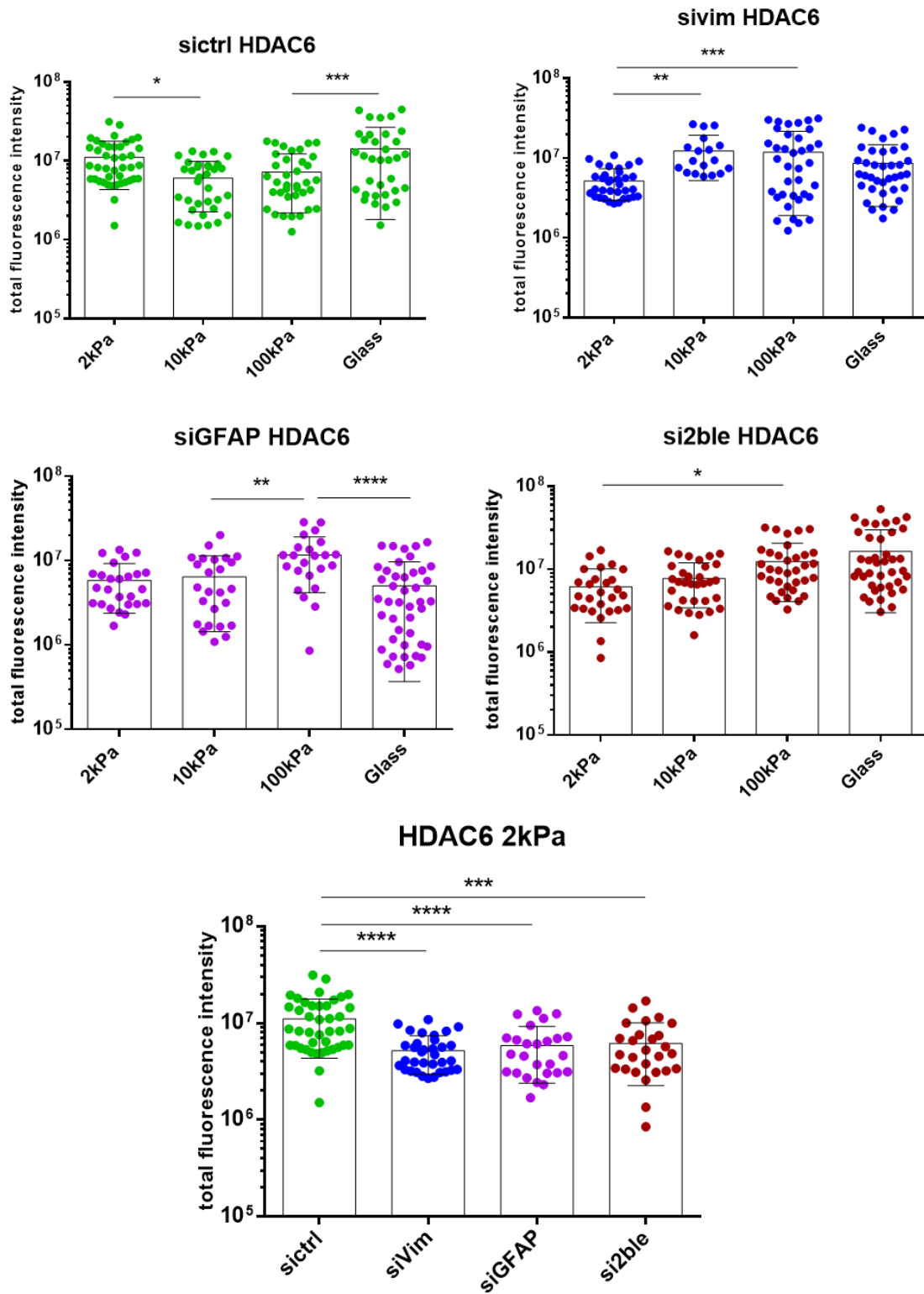


Figure 2 HDAC6 expression in different rigidity. Cells were transfected with siRNA against IFs and plated substrates of different rigidities. They were then fixed and stained and then quantified for HDAC6. N=2. . Statistical analyses done using One-way ANOVA followed by Tuckey's multiple comparison test (** $p < 0.01$; *** $p = 0.0001$; **** $p < 0.0001$).

Appendix 4

Script Fiji macro for fluorescence quantification around the nucleus, written by Dr. Emma van Bodegraven.

```
run("Bio-Formats Importer");
dir1 = getDirectory("SelectDirectory");

while (nImages>0) {

    selectImage(nImages);
        imageTitle=getTitle();//returns a string with the image title
        run("Split Channels");
        selectWindow("C1-"+imageTitle);
        //setTool("wand");
        task = "Click on cell to outline";
        msg = "DubbleClick on wand to adjust size and press OK";
        waitForUser(task, msg);
        roiManager("Add");
        roiManager("Select", 0);//roi 0 can measure intensity 'underneath' nucleus
        run("Make Band...", "band=2.5");
        roiManager("Add");//makes roi 1 and measure first peri-nuclear band
        roiManager("Select", 0);
        run("Enlarge...", "enlarge=2.5");
        run("Make Band...", "band=2.5");
        roiManager("Add");//makes roi 2 and measure second peri-nuclear band
        selectWindow("C3-"+imageTitle);
        //setTool("wand");
        task = "Click on cell to outline";
        msg = "DubbleClick on wand to adjust size and press OK";
        waitForUser(task, msg);
        roiManager("Add");//makes outline of the cell and roi 3
        roiManager("Select", 3);//roi 3 can measure intensity of whole cell
        run("Enlarge...", "enlarge=-2.5");
```

```

run("Make Band...", "band=2.5");
roiManager("Add"); //roi 4 and measure first peripheral band
roiManager("Select", 3);
run("Enlarge...", "enlarge=-5");
run("Make Band...", "band=2.5");
roiManager("Add"); //roi 5 and measure second peripheral band
//now create an roi in the leftover part of the cell
//make roi of outside second perinuclear roi
roiManager("Select", 0);
run("Enlarge...", "enlarge=5");
run("Make Inverse"); // make roi of all outside this region
roiManager("Add"); //makes roi number 6
roiManager("Select", 3);
run("Enlarge...", "enlarge=-5"); // make roi for all within cell after second peripheral
band
roiManager("Add"); //makes roi number 7
roiManager("select", newArray(6,7));
roiManager("AND");
roiManager("Add"); //makes roi number 8 and measures middle of the cell
//start measurement vim = C3
selectWindow("C2-"+imageTitle);
roiManager("Select", 0);
run("Flatten");
saveAs("Tiff", dir1+"roi0"+imageTitle);
close();
selectWindow("C2-"+imageTitle);
roiManager("Select", 0);
run("Measure");
roiManager("Select", 1);
run("Flatten");
saveAs("Tiff", dir1+"roi1"+imageTitle);
close();
selectWindow("C2-"+imageTitle);

```

```
roiManager("Select", 1);
run("Measure");
roiManager("Select", 2);
run("Flatten");
saveAs("Tiff", dir1+"roi2"+imageTitle);
close();
selectWindow("C2-"+imageTitle);
roiManager("Select", 2);
run("Measure");
roiManager("Select", 3);
run("Flatten");
saveAs("Tiff", dir1+"roi3"+imageTitle);
close();
selectWindow("C2-"+imageTitle);
roiManager("Select", 3);
run("Measure");
roiManager("Select", 4);
run("Flatten");
saveAs("Tiff", dir1+"roi4"+imageTitle);
close();
selectWindow("C2-"+imageTitle);
roiManager("Select", 4);
run("Measure");
roiManager("Select", 5);
run("Flatten");
saveAs("Tiff", dir1+"roi5"+imageTitle);
close();
selectWindow("C2-"+imageTitle);
roiManager("Select", 5);
run("Measure");
roiManager("Select", 8);
run("Flatten");
saveAs("Tiff", dir1+"roi8"+imageTitle);
```

```

close();
selectWindow("C2-"+imageTitle);
roiManager("Select", 8);
run("Measure");
//new channel
selectWindow("C4-"+imageTitle);
roiManager("Select", 0);
run("Flatten");
saveAs("Tiff", dir1+"2roi0"+imageTitle);
close();
selectWindow("C4-"+imageTitle);
roiManager("Select", 0);
run("Measure");
roiManager("Select", 1);
run("Flatten");
saveAs("Tiff", dir1+"2roi1"+imageTitle);
close();
selectWindow("C4-"+imageTitle);
roiManager("Select", 1);
run("Measure");
roiManager("Select", 2);
run("Flatten");
saveAs("Tiff", dir1+"2roi2"+imageTitle);
close();
selectWindow("C4-"+imageTitle);
roiManager("Select", 2);
run("Measure");
roiManager("Select", 3);
run("Flatten");
saveAs("Tiff", dir1+"2roi3"+imageTitle);
close();
selectWindow("C4-"+imageTitle);
roiManager("Select", 3);

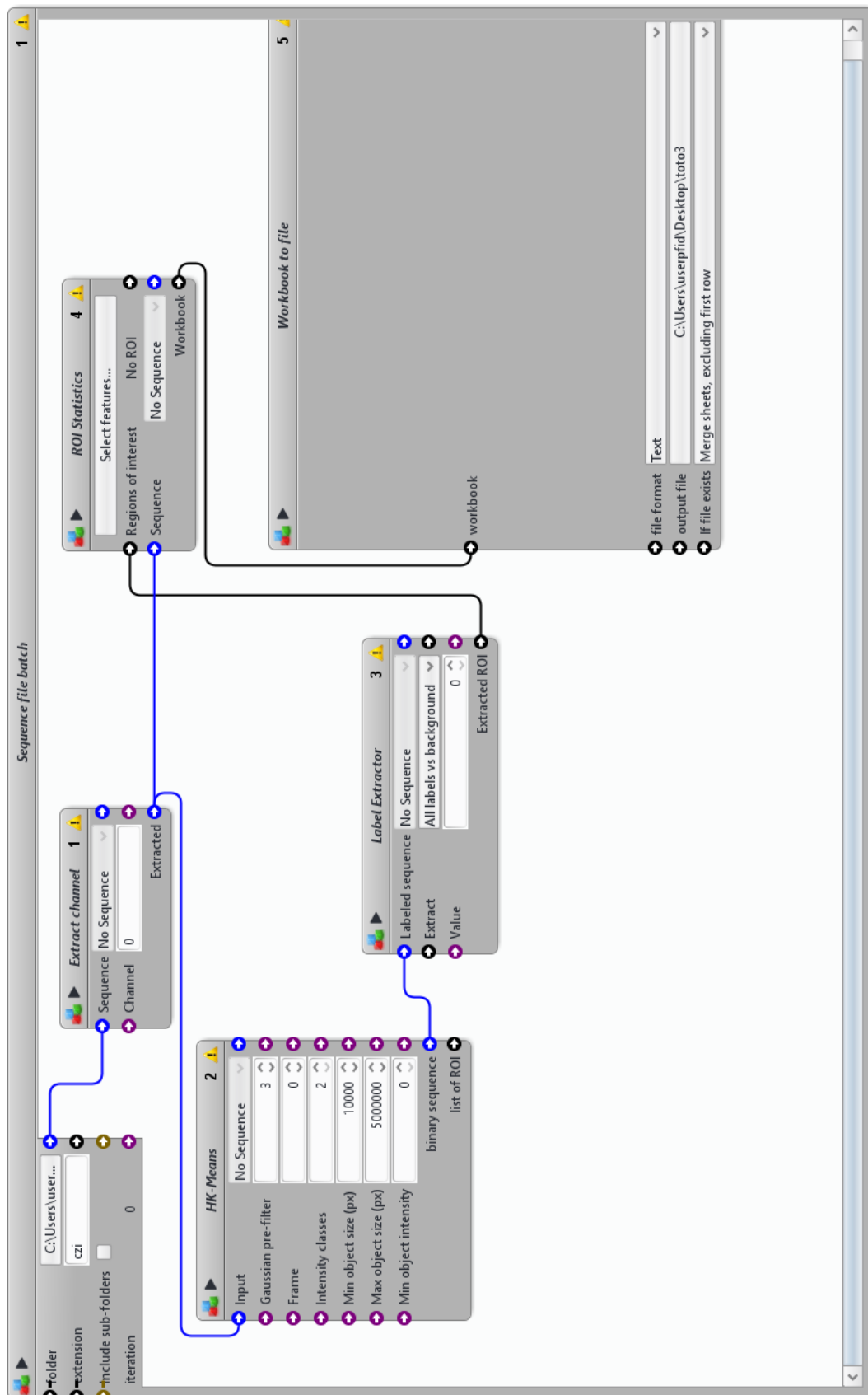
```

```
run("Measure");
roiManager("Select", 4);
run("Flatten");
saveAs("Tiff", dir1+"2roi4"+imageTitle);
close();
selectWindow("C4-"+imageTitle);
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roiManager("Select", 5);
run("Flatten");
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close();
selectWindow("C4-"+imageTitle);
roiManager("Select", 5);
run("Measure");
roiManager("Select", 8);
run("Flatten");
saveAs("Tiff", dir1+"2roi8"+imageTitle);
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selectWindow("C4-"+imageTitle);
roiManager("Select", 8);
run("Measure");
roiManager("reset");
selectWindow("C1-"+imageTitle);
close();
selectWindow("C2-"+imageTitle);
close();
selectWindow("C3-"+imageTitle);
close();
selectWindow("C4-"+imageTitle);
close();
```

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}
```


Appendix 5

Script Icy, calculation of volumes



Publication: “Intermediate filaments”

This appendix consists of the review published in Current Biology. Parts of the review were used in the introduction.

<https://doi.org/10.1016/j.cub.2021.04.011>

alleles that have been sequenced, most are nonsense mutations and the seven missense mutations identified were all in the FH2 domain, compromising actin assembly to different degrees. In this case, overactive Capu may be tolerated because the formin is autoinhibited and not essential, but loss of activity is detrimental to egg development. The mammalian homolog of Capu, Fmn2, is essential in the developing egg, with its loss leading to infertility in mice and likely in humans as well.

Formin functions outside of actin assembly

The length of this section should not belie its importance. Formins can have additional activities aside from actin assembly. Many, but not all, formins bind to the sides of actin filaments and most of these bundle filaments, creating larger assemblies. At least one formin, INF2, severs actin filaments in addition to building them. Interestingly, several formins, either alone or in conjunction with microtubule-binding proteins, stabilize microtubules. They may crosslink and align actin filaments with microtubules, making formins central to overall cytoskeletal organization in some cases. Finally, some formins have additional conserved domains, such as PDZ, pleckstrin homology (PH), and WH2 domains, which likely contribute to specificity of function by determining localization or additional binding proteins. This variety of capabilities is part of how formins contribute to a broad array of structures and functions.

Remaining questions

There is more to learn. On the biochemical front, nucleation is still poorly understood. We have a firmer grasp on elongation but there is plenty to learn about the elements that control gating, processivity, and potential crosstalk between the FH1 and FH2 domains. For example, how does a formin remain bound to a continuously growing filament end? On the cell biological front, roles for many formins are yet to be determined. In some cases, this may be complicated by crosstalk and/or compensation. For example, researchers have long sought the mechanism of actin filament formation and regulation within the sarcomere. Many proteins have been identified, including at least three formins. With

advances on these two fronts we will begin to understand the link between the specific activity of a formin and its function. Finally, many proteins are known to have strong effects on different elements of actin assembly, but clear common themes have yet to be identified: there are certainly more formin-interacting proteins to be found and studied. In conclusion, all nine metazoan subfamilies of formins share common activities in terms of nucleation and elongation, but are also distinguished by activity level, localization, and cell-type-specific expression, which are probably among many other aspects that remain to be discovered!

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Primer Intermediate filaments

Gaëlle Dutour-Provenzano^{1,2}
and Sandrine Etienne-Manneville^{1,3*}

Cell morphology, architecture and dynamics primarily rely on intracellular cytoskeletal networks, which in metazoans are mainly composed of actin microfilaments, microtubules and intermediate filaments (IFs). The diameter size of 10 nm — intermediate between the diameters of actin microfilaments and microtubules — initially gave IFs their name. However, the structure, dynamics, mechanical properties and functions of IFs are not intermediate but set them apart from actin and microtubules. Because of their nucleotide-independent assembly, the lack of intrinsic polarity, their relative stability and their complex composition, IFs had long been overlooked by cell biologists. Now, the numerous human diseases identified to be associated with IF gene mutations and the accumulating evidence of IF functions in cell and tissue integrity explain the growing attention that is being given to the structural characteristics, dynamics and functions of these filaments. In this Primer, we highlight the growing evidence that has revealed a role for IFs as a key element of the cytoskeleton, providing versatile, tunable, cell-type-specific filamentous networks with unique cytoplasmic and nuclear functions.

Elucidating IF physiological functions

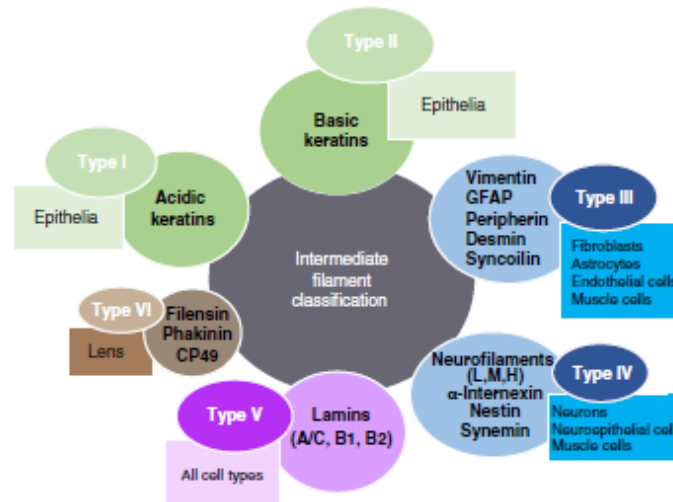
IFs are generated by a wide variety of proteins encoded by 73 genes in humans. With the exception of nuclear lamins, which are found in all cells of multicellular organisms, the expression of IF proteins, such as keratins, desmin or vimentin, is cell-type specific and also varies during cell differentiation (Figure 1). IF-related diseases affect particular tissues or organs that mostly correspond to the sites of expression of the particular IF proteins. More than 90 pathologies, including the so-called laminopathies, keratinopathies and desminopathies, have been associated with mutations in IF proteins. Mutation of desmin, which is specifically expressed



in muscle cells, impacts muscle function causing skeletal myopathies or cardiomyopathies. In contrast, mutations in keratins, which form a large family of IF proteins differentially expressed as sets of one, two, or three pairs in diverse epithelia, give rise to several skin, hair, lens and liver disorders.

In contrast, knockout animal models have not always provided clear insights into IF functions, possibly because of the complexity of the protein expression patterns and functional redundancy. A major initial publication in 1994 from Colucci-Guyon and colleagues claimed that loss of vimentin, a major IF protein expressed in all mesenchymal cells, did not have any *in vivo* phenotypic effect. However, further studies have shown that vimentin actively participates in a wide range of cell, tissue and organ functions. Loss of vimentin induces a decrease in Bergmann glial cells and Purkinje cells in the cerebellum, together with a deficit in motor coordination, compromised endothelial integrity with increased arterial stiffness, and delayed lymphocyte and fibroblast migration during wound healing. Vimentin is also critical in pathological situations, for instance providing protection against bacterial meningitis.

Mouse models with various IF deficiencies confirm the essential role of IFs in tissue physiology. Loss of keratins induces early embryonic lethality, requiring the use of conditional deletion to gain further insight into their function in adults. Mice lacking glial fibrillary acidic protein (GFAP), an IF protein expressed by glial cells in the central nervous system, are more sensitive to percussive head injuries and display altered astrogliosis and wound healing. Other examples include desmin, deletion of which dramatically affects skeletal, smooth and cardiac muscle formation, and synemin, which plays a structural role in bone formation and mechanical resistance. These *in vivo* observations show that loss of IF proteins causes alterations that are different from those observed in human diseases associated with mutations in IF proteins. In fact, IF-related diseases are caused by mutations that generally lead to the reorganization but not the loss of the IF network. For instance, heterozygous mutations in the type III GFAP gene that cause Alexander disease engender the accumulation of IF proteins in



Current Biology

Figure 1. Intermediate filament classification.

Type I and II proteins correspond respectively to acidic and basic keratins, the most abundant IF proteins, which associate in obligate heteropolymers that are highly expressed in epithelial cells. Type III proteins are expressed in a variety of cell types, including fibroblasts, and endothelial, glial and muscle cells, and comprise vimentin, desmin, glial fibrillary acidic protein (GFAP), as well as peripherin. Type IV proteins, including nestin, synemin, α -internexin, syncoilin and neurofilament proteins, are mainly expressed in progenitor or differentiating cells and also in neuronal and glial cells. Type V proteins correspond to the ubiquitously expressed lamins, which contain a nuclear localization sequence and a CAAX box motif that targets the protein to the inner nuclear membrane. Finally, type VI represents a group of very divergent proteins, including the lens-specific IF proteins (Bfsp1/filensin, Bfsp2/phakinin and CP49).

proteinaceous aggregates called Rosenthal fibers in astrocytes (Figure 2). More specific mouse models expressing mutated IF proteins have confirmed that the altered organization of the IF network plays a key role in these pathologies. Finally, IF-related diseases and *in vivo* models point to an essential role for IFs in tissue mechanical resistance and tissue integrity.

Human genetic disorders demonstrate the importance of IF physiological functions. They reveal the need to understand the role of IFs at the cellular and tissue scales but also underline the complexity of the task. Because of the high number of possibly redundant IF proteins, the wide variety of expression patterns, the intrinsic stability of IFs, and the lack of specific inhibitors, the identification of IF functions has lagged behind that of actin and microtubules. Our understanding of IF roles at the cellular and tissue scales has emerged from the careful and still ongoing

characterization of IF molecular structure. Building on the unique structural properties of IF proteins, the characterization of IF mechanical properties and the progressive elucidation of the regulatory mechanisms controlling IF dynamics in cells, we now have a better grasp of the extent of the cellular functions of IFs that can eventually explain IF-related pathologies.

Composition, assembly, structure and intrinsic properties of IFs

IF proteins

IF proteins are derived from a common ancestor of the more ubiquitously expressed nuclear lamins, traced back to organisms outside of the metazoan lineage, like the slime mold *Dictyostelium*. IF proteins have a uniform, global structure based on a common central domain. The first X-ray crystal structure of an IF protein — keratin — was reported in 1932 by William Astbury and led, through Linus Pauling's α -helix

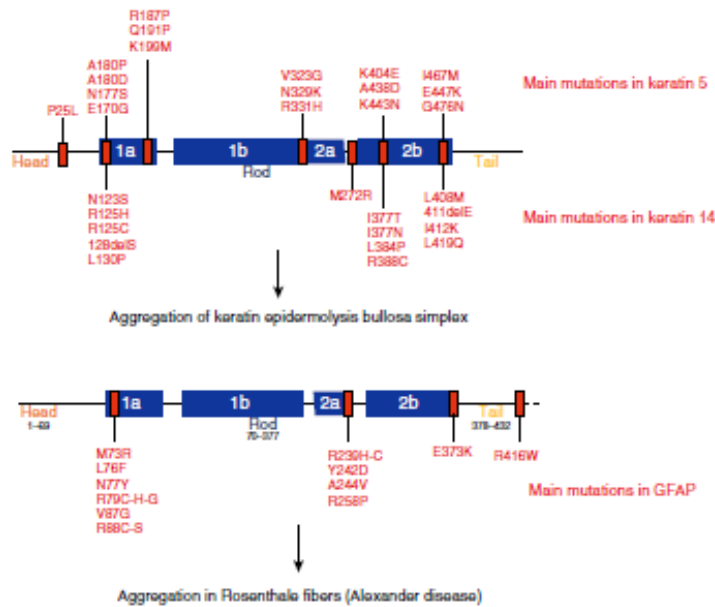


Figure 2. IF mutations are responsible for human diseases. The schematics show the main mutations (in red) observed in keratin 5 and keratin 14 and in glial fibrillary acidic protein (GFAP) that give rise to epidermolysis bullosa simplex and Alexander disease, respectively.

model, to the prediction of the coiled-coil structure of keratin by Francis Crick in 1952. This common α -helical rod domain of approximately 310 amino acids is present in all IF proteins. It is flanked by head and tail domains of diverse size and structure that characterize each IF protein and allow them to interact with specific intracellular partners. Depending on their distinct rod domain sequence and terminal domain functions, IF proteins are classified into six categories (Figure 1) that differ in their biochemical properties and their ability to associate with one another.

IF assembly

To form filaments, IF proteins first assemble into dimers through the rod domain of two monomers that are twisted together in a coiled-coil structure. For cytoplasmic IFs, dimers then associate in an antiparallel fashion to form apolar tetramers that constitute the soluble building blocks of the filaments. Eight tetramers join laterally to give rise to a unit length filament (ULF) of approximately 50 nm in length (Figure 3). ULFs assemble end-to-end

to constitute a filament with a diameter of 16 nm that is radially compacted to form the classical 10 nm diameter filament observed in cells. Notably, nuclear IFs composed of lamins form by concomitant lateral and longitudinal association of the dimers, generating filaments with variable diameter and length. In all cases, IFs, unlike actin microfilaments and microtubules, polymerize independently of nucleotides or protein nucleators. Instead, IF dynamics involve fusion and fission of ULFs or small pre-existing filaments (also called squiggles) and the exchange of subunits. The extent of such fusion/fission and exchange events in cells is not clear, however, and their regulation remains to be elucidated.

The structural diversity of IFs has also not been fully characterized. While keratins exclusively form heterodimers between type I and type II proteins, it is not known how type III IF proteins self-assemble to form homopolymers and also co-polymerize with other type III or type IV proteins. This co-assembly can involve four or five different IF proteins, as observed in astrocytes where

GFAP, vimentin, nestin and synemin are observed in the same filaments or in neurons where the neurofilament proteins NF-L, NF-M, NF-H can interact with α -internexin or peripherin. It remains to be studied whether different IF proteins assemble in heteropolymers by forming common or distinct dimers, tetramers or ULFs that then assemble to form a single filament.

To the initial diversity in IF genes must also be added the large number of splice variants. Keratin, vimentin, desmin, lamin, peripherin, synemin and GFAP are all alternatively spliced, and some of them have multiple splice variants, like GFAP, which has more than 10 splice variants that can have different effects on IF polymerization. Finally, a variety of post-translational modifications such as farnesylation, glycosylation, transglutamination, acetylation, ubiquitination, sumoylation and phosphorylation can occur on IF proteins (Figure 4). The complex combination of post-translational modifications decorating IFs is likely associated with a wide range of dynamic and functional properties.

IF mechanical properties

Because of their core structure built on the rod domains and their nucleotide- and nucleator-independent mode of assembly, IFs are extremely flexible and stretchable and are also resistant to low temperatures, high ionic concentrations, and mechanical breakage. For instance, desmin IFs found in muscle cells can be stretched *in vitro* to about 2.5 times their original length before breakage. When subjected to force, the coiled-coil α -helical domain of each monomer can be individually stretched to eventually transition to a β -sheet conformation. The remaining α -helical domains within a ULF allow it to return to its original length during relaxation. When subjected to large deformations *in vitro*, IFs have an extraordinary ability to stiffen and can therefore withstand large strains without breaking. Strain-stiffening is also observed with lamin filaments, which deform reversibly at a few hundred piconewtons and stiffen at nanonewton forces. The stiffening of the filament is attributed to an increased pulling force on β -sheets. The softness of IFs observed under compression also leads to a progressive stiffening; however, unlike actin filaments and

microtubules, these filaments cannot buckle.

IF structure is at the core of the unique mechanical properties of these filaments *in vitro*. As we are still missing some essential information about the structure of complex IF polymers in cells, we can only assume that IF composition, alternative splicing and post-translational modifications influence the mechanics of these filaments. That said, analyses of IF functions in cells are progressively revealing how IF networks participate in cell and tissue integrity.

IFs as integrators of intracellular space

Resilient intracellular scaffolds

An important characteristic of most IF-related diseases is that the gene mutations do not result in loss of the IF protein but instead induce the aggregation or the collapse of the network in a perinuclear area. This points to an essential role of IF network organization within cells. In mammalian cells, IFs form at least two distinct intracellular networks: the cytosolic and the nuclear networks (Figure 3). Because IF proteins of different types cannot always co-assemble, cells can sometimes display two distinct cytoplasmic IF networks. For instance, distinct type III IFs, like vimentin IFs and keratin IFs, can be found in epithelial cells (Figure 3). Cytoplasmic and nuclear IFs form scaffolding structures that resist and also adapt to the physical challenges of their environment. Cytoplasmic IFs extend between the periphery of the cell and the nucleus and often surround the nucleus, forming a so-called perinuclear cage. Nuclear lamin polymers form the nuclear lamina, a protein meshwork that supports the nuclear envelope structure and tethers heterochromatin to the nuclear periphery.

Because of their robustness and elastic properties, IFs are excellent stress absorbers and key for the mechanical resilience of the cell. This is reflected by their essential contribution to the regulation of nuclear and cell stiffness under deformation. In response to low levels of deformation, IFs make cells compliant, whereas in response to high levels of stress and deformation, IFs provide cells with mechanical strength and stiffness. The expression pattern of IF proteins in a given cell type may tune the cellular mechanical properties

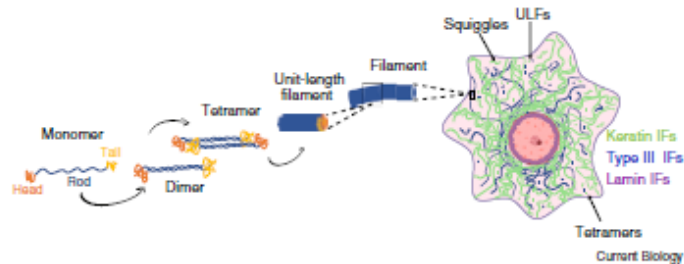


Figure 3. IF assembly.

Monomers of IFs assemble into a dimer through an interaction between their rod domains. Dimers assemble in an antiparallel manner to form soluble tetramers, which in turn assemble into unit length filaments (ULFs) that, via end-to-end binding, elongate into filaments. Soluble tetramers, ULFs, and squiggles, which correspond to short IFs and long IFs, can all be found in cells.

to the specific tissue environment. Differences in amino acid sequence, as observed between different IF proteins or mutant variants, or post-translational modifications can change the charge and hydrophobicity of the IF proteins. In turn, this affects the stretching and sliding of monomers within the filaments and may account for the variation in flexibility observed between IF types. As an example, neurofilaments tend to be much softer than other IFs, which may be related to their expression in brain tissues, which are particularly soft. IF function in cell mechanics is particularly important in tissues subjected to frequent mechanical stress, such as striated muscles, heart, skin and blood vessels. Mutations in keratin 5 and keratin 14, causing epidermolysis bullosa simplex (Figure 2), disrupt the mechanical integrity of the epidermis and cause blistering, and desmin mutations affect the integrity of skeletal and cardiac muscle tissues.

Mechanical and functional support for intracellular organelles

The intracellular scaffolds formed by IFs influence organelle position and dynamics by affecting cytoplasmic viscosity. However, IFs have a more direct role in the organization of the cellular space, as they also interact directly with intracellular organelles. As a consequence, IFs are responsible for the positioning and the number of autophagosomes and lysosomes during autophagy and play a role in the distribution of melanosomes. They also influence both anterograde and retrograde vesicular transport via their

effects on the positioning of intracellular organelles. Alteration of the IF network or mutations in IF proteins affect not only the distribution of organelles but also their associated signaling pathways, eventually leading to loss of specific organelle functions. The role of IFs in the localization, structure and function of intracellular organelles is best illustrated by their interaction with mitochondria. IFs surround mitochondria either by confinement or by binding the mitochondrial membrane via the protein plectin. Mitochondria fulfill essential roles in ATP production, metabolic regulation, calcium signaling, generation of reactive oxygen species (ROS), which are products of oxidative stress, and apoptosis. Defects in mitochondrial distribution, morphology and function are observed in several IF-related diseases such as Charcot-Marie-Tooth neuropathy or epidermolysis bullosa simplex. By controlling mitochondrial structure and cristae integrity, preventing proton leaks and maintaining transmembrane potential, cytoplasmic IFs prevent the generation of ROS, shedding light on the causes of oxidative stress observed in these disorders.

Both nuclear and cytoplasmic IFs interact with the nuclear envelope through the linker of nucleoskeleton and cytoskeleton (LINC) complex, an assembly of proteins anchoring the cytosolic cytoskeleton to the intranuclear meshwork of lamins. The cage-like structure of IFs around the nucleus actively participates in the control of nuclear shape and positioning. The dense and elastic cytoplasmic IF network contributes to the transmission

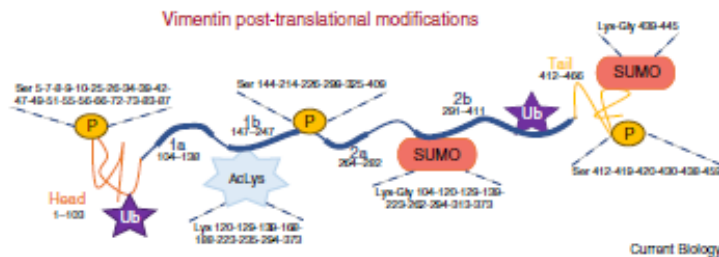


Figure 4. IF post-translational modifications. The major post-translational modifications, including phosphorylation, sumoylation, acetylation and ubiquitination, that are known to affect vimentin are shown. As for all IF proteins, post-translational modifications occur throughout the vimentin protein and affect the dynamics, mechanics and biochemical properties of the resulting filaments.

of external forces applied at the cell cortex to the nucleus. In skeletal muscle, nuclei increase their length and aspect ratio upon cell stretching. The stretching capability of the nucleus relies on the desmin network, which transmits force towards the myofibrillar nuclei. The IF perinuclear cage also protects the nucleus from excessive forces. When cells migrate through tight constrictions that squeeze the nucleus, vimentin IFs prevent nuclear rupture and DNA damage. Vimentin levels also control chromatin condensation, suggesting a direct link between nuclear deformations and the control of gene expression. The possibility that cytoplasmic IFs enter the nucleus is a matter of debate. Keratins, such as keratin 17, have been shown to interact with nuclear proteins and impact nuclear morphology, histone modification, chromatin organization, gene expression, and proliferation in epithelial cells.

The intranuclear meshwork of lamins plays a fundamental role in nuclear shape and mechanics and the elastic capabilities of lamins are crucial in upholding nuclear integrity during cell compression. Lamins also affect chromatin dynamics in the interphase nucleus and may thereby influence nuclear functions. Because of their tight interaction with the nuclear envelope, and their unique mechanical properties, IFs serve to protect nuclear integrity and probably also influence the mechanosensitive organization and functions of the nucleus. In skeletal muscle cells lamin A mutations related to muscular dystrophy cause transient rupture of the nuclear envelope, resulting in DNA damage, activation of the DNA damage response, and reduced cell

viability. Moreover, like cytoplasmic IFs, lamins are also important in protecting the cell and the nucleus against oxidative stress: a higher percentage of ROS is found in patient fibroblasts expressing mutated lamin A following induction of oxidative stress.

IFs as integrators of cell mechanical properties

Cytoskeletal connections
The structural and mechanical functions of IFs are reinforced by their tight connection and crosstalk with the actin and microtubule networks. This is perfectly illustrated in muscle fibers, where desmin filaments connect the Z-discs to the plasma membrane, mitochondria and nuclei to increase the mechanical resilience of muscle cells. In addition to a possible direct interaction with actin microfilaments, a wide variety of cytoskeletal crosslinkers connect IFs to actin and microtubules: these include plectins, β -crystallin, fimbrin, filamin A, adenomatous polyposis coli (APC), as well as a number of cytoskeletal motors.

The impact of IFs not only is a result of their physical interactions with actin and microtubules but also involves their function in key signaling pathways controlling, for instance, RhoA activity and cell contractility. IFs influence the dynamics and organization of the microtubule and actin networks. Accumulating evidence points to IFs as major regulators of actin stress fibers and actomyosin-mediated forces, although the effects vary with cell type, possibly as a consequence of differences in IF composition. Although no major change in the global organization of the microtubule network has been observed in IF-depleted cells,

IFs can modulate the dynamics of microtubules. Vimentin IFs interact with microtubules and serve as a template to subtly direct microtubule growth. Microtubules that are directly associated with vimentin are also more resistant to microtubule-depolymerizing drugs like nocodazole. Whether all types of IF have the same effect on the other cytoskeletal networks awaits further investigation. However, it is already clear that keratin and vimentin networks behave very differently in relation to microtubules, as microtubule depolymerization induces the perinuclear collapse of the vimentin IF network without affecting keratin IF organization. Overall, it is likely that IF composition or post-translational modifications influence cell contractility and motile behavior and may be key to the adaptation of various cell types to the specific, and possibly evolving, mechanical properties of their environment.

Interactions with adhesive structures

The scaffolding structure formed by cytoplasmic IFs, together with the other cytoskeletal networks, is connected to the extracellular microenvironment. IFs interact with, and also influence cell-cell and cell-matrix adhesive structures. This was initially illustrated by the association of keratin filaments with desmosomes via desmoplakin and with hemidesmosomes via bullous pemphigoid antigen 1 and 2 (BPAG1 and 2). Keratin IFs stabilize hemi-desmosomes and desmosomes, which are essential for the cohesion of epithelial tissues. The stabilizing role of IFs on cell adhesions is not limited to epithelial cells. IFs are involved in the stability of gap junctions in cardiomyocytes and of adherens junctions in endothelial cells. IFs also associate with focal adhesions in many cell types, including fibroblasts, endothelial cells and astrocytes. The organization of IFs at focal adhesions and the exact molecular link that connects IFs to integrins are not entirely clear and likely vary depending on the IF composition and on the integrin involved. One specific IF protein, synemin, directly interacts with several focal adhesion proteins, such as talin, vinculin and zyxin. In the absence of synemin, plectin (more specifically plectin 1f) is involved in the association of vimentin and desmin with integrins. IFs tend to

stabilize or reinforce focal adhesions in immobile cells. However, the molecular mechanisms physically and functionally bridging IFs to focal adhesions need to be further investigated, focusing on the specific relationship of each IF protein with various integrins and focal adhesion proteins.

IFs as integrators of intracellular signaling

IFs not only serve as a structural scaffold but also form a molecular scaffold that connects with signaling pathways to influence cell behavior in physiological and pathological situations. The connection of IFs with intracellular structures parallels the ability of these filaments to influence cellular functions. While focal-adhesion-mediated signaling influences the organization of the IF network, IFs can in turn influence the dynamics of focal adhesions. They participate in focal-adhesion-associated signaling, as shown for vimentin, which regulates the expression level and the localization of focal adhesion kinase (FAK) and the Rac1 guanine nucleotide exchange factor Vav2. In addition to their role in controlling actomyosin contractility, vimentin and more generally type III IFs facilitate cell migration and invasion of mesenchymal cells by controlling the dynamics and distribution of focal adhesions. Depending on the cell type, IFs have different effects on cell migration that may be explained by the difference in IF proteins or integrin expression patterns.

Mirroring their protective role against mechanical stresses, the signaling functions of IFs are also involved in cellular survival by promoting cell-cycle progression, maintaining organelle homeostasis and protecting against apoptosis. Most IF proteins, including keratins, GFAP, vimentin and neurofilament proteins, interact with 14-3-3 proteins. The 14-3-3 family of proteins forms a major class of molecular chaperones, binding several kinases including Raf, protein kinase C (PKC), c-Bcr, Bcr-Abl and phosphatidylinositol 3-kinase, thereby regulating their activity, localization or stability. By controlling 14-3-3 localization, IFs are well positioned to influence a wide range of vital regulatory processes, such as mitogenic signal transduction, apoptotic cell death, and cell-cycle control. 14-3-3 interactions with target proteins may

participate in the formation of protein aggregates, such as those observed in neurodegenerative diseases, and may regulate pathogenic processes. Other signaling functions have been described for keratin IFs. Keratin 8–18 IFs interact with the cytoplasmic tail of tumor necrosis factor (TNF) receptors to influence their signaling and protect cells from apoptosis. The same keratin 8–18 IFs have also been shown to interact with and activate Notch1 and promote epithelial differentiation in the large intestine. In the case of Notch regulation, vimentin can also play a crucial regulatory role: in endothelial cells, vimentin regulates Notch signaling strength and arterial remodeling in response to hemodynamic forces, suggesting that several IF proteins may contribute to the regulation of the Notch pathway. In the nucleus, lamins control nucleocytoplasmic transport and gene expression: increased lamin B1 expression in huntingtin-mediated neurodegeneration affects the chromatin domains associated with lamins, chromatin accessibility and transcriptional regulation.

We are probably only scratching the surface when considering IF involvement in biochemical signaling. How the composition of IFs can modulate intracellular signaling is also a broad question that systematic proteomic analysis of IF molecular partners may help to answer. In addition, recent evidence reveals a role for soluble IF proteins in IF signaling functions. Phosphorylated soluble lamins interact with gene enhancer regions to control gene expression, suggesting that local or partial depolymerization of IFs may provide soluble IF proteins that serve as signaling intermediates.

Dynamic control of IF functions

Control of IF protein expression

Changes in IF protein expression can modulate the mechanical properties of the network and consequently of the cells and tissues. An accumulation of 100–1,000 times the normal level of keratin filaments in the cornified layer of the skin generates a material with an elastic modulus in the megapascal range. Within the blood vessel network, vimentin levels are present at levels proportional to the size of the vessel. Indeed, with increasing shear stress due to heavier blood

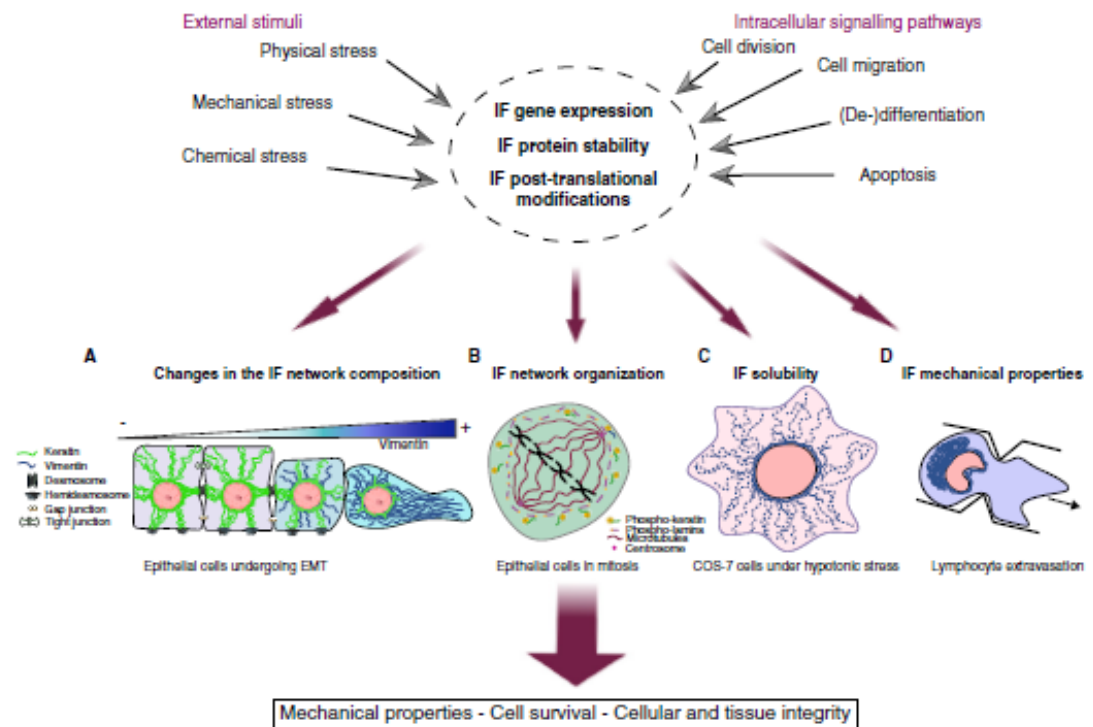
flow in large arteries, the endothelial cells and vascular smooth muscle cells need higher resistance to the pressure. Changes in the composition of the network generally parallel cell differentiation and allow for important but relatively slow modifications of the cell and tissue mechanics. During epithelial–mesenchymal transition, vimentin expression increases and facilitates cell shape changes and cell invasion (Figure 5A).

IF protein expression is controlled at the transcriptional level but also via regulation of IF protein stability. Ubiquitination targets IF proteins for degradation, a process that is crucial for the correct turnover of the IF network, as illustrated by giant axonal neuropathies, in which mutations of the E3 ligase targeting factor gigaxonin lead to an accumulation of IFs in the central nervous system. As yet, there is no clear evidence that the network composition can be tuned at a subcellular level, and therefore such changes in protein expression may only allow for global long-term changes in the properties of the network.

Temporal control

Active rearrangements of the IFs, including polymerization, depolymerization, aggregation, transport or bundling of filaments, modify the mesh size and the mechanical properties of the network. In hair, nail, hoof and quill, keratin IFs are crosslinked by disulfide bonds giving rise to materials with an elastic modulus in the gigapascal range. In epithelial cells, the mesh size of the keratin network tends to increase towards the cell periphery, providing a less elastic network, which might facilitate cell protrusion and motility. Upon shear stress, the mesh size becomes more homogeneous throughout the cell and generates a more elastic and protective intracellular structure. The control of the mesh size indicates that intracellular signals can modify IFs as cells change behavior or adapt to different microenvironments. Dramatic rearrangements of the IF network are indeed observed as cells respond to external stresses, divide, migrate or die (Figure 5).

IF organization drastically changes when cell morphology and functions require an adaptation of cell mechanical properties and the reorganization of



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Figure 5. Extracellular and intracellular signals lead to changes in IFs to ensure cellular mechanical stability and tissue integrity. Cells are subjected to external stimuli and intrinsic changes that modify the expression, stability and post-translational modification of IFs. These changes affect the composition, organization, and the mechanical and biochemical properties of the IF network. (A) During epithelial–mesenchymal transition (EMT), keratin in epithelial cells is progressively replaced by a vimentin network as the cells transition to a migrating mesenchymal type, facilitating tissue invasion. (B) The cytoplasmic and nuclear IF network is modified during cell division via the phosphorylation of cytoplasmic IFs (here keratins) and lamins. (C) Phosphorylation of IFs leads to the solubilization of the network in response to osmotic shock. (D) The organization of the vimentin IF network is dramatically altered as lymphocytes migrate between endothelial cells during extravasation.

the cytoplasm. During cell division (Figure 5B), the redistribution of the IF network affects the organization of the cortical actin network and contributes to mitotic progression. Remodeling of the IF network during cell-cycle progression and mitosis is mostly triggered by phosphorylation of IF proteins, in particular by cyclin-dependent kinases, altering their filamentous organization and rendering them more (or less) soluble. Phosphorylation can similarly promote the disassembly of the nuclear lamina and thus promote nuclear envelope breakdown. External stress, including heat stress, osmotic stress, and mechanical stress, generally induces the hyperphosphorylation of IF proteins and the reorganization of the network (Figure 5C). This is frequently

associated with the binding of IFs to heat shock proteins, with different IFs binding to overlapping or specific heat shock proteins. These observations led to the hypothesis that IFs may protect the cell from stress-induced death by passively buffering the activity of stress-activated kinases. However, our more recent understanding of the functions of the IF network and their regulation by intracellular signaling suggests that stress-induced changes in IF organization may play an active role in adapting cell mechanics, intracellular organization and functions of key organelles to maintain cell homeostasis. During apoptosis, the cytoplasmic and nuclear IF networks are completely disrupted and collapse in large aggregates as proteins are irreversibly

cleaved by caspases, and this allows the cell to shrink and die. As a result of changes in IF composition, reversible or irreversible post-translational modifications and cytoskeletal coupling, the IF network adapts to major changes in the cell environment or cell behavior. More subtle rearrangements of the IF network can occur at a subcellular level and contribute to local adjustment of the scaffolding properties of the IF network.

Spatial control

Signaling cascades downstream of membrane receptors can locally influence the dynamics and properties of IFs; for example, Rho GTPases and their effectors PAK and ROCK modulate IF protein phosphorylation, and regulation of Rho signaling downstream of integrins

can alter the local organization of the IF network. In migrating epithelial cells, the global redistribution of polymerization sites towards the leading edge facilitates the generation of new filaments and organizes the turnover of the network. In contrast, in migrating astrocytes the polarization of the IF network relies on increased kinesin-mediated transport of vimentin and GFAP-containing IFs towards the leading edge, coupled to a local integrin-dependent Cdc42-driven inhibition of their dynein-mediated retrograde transport. Focal-adhesion-mediated signaling also regulates vimentin phosphorylation. Fibroblasts plated on soft substrates show increased vimentin phosphorylation associated with an increase in the soluble pool of vimentin, suggesting an adaptation of the properties of the network to the mechanical characteristics of the cell microenvironment. Cell stretching has been recently shown to increase the tension of keratin fibers, thereby controlling their interaction with C-terminal tensin-like protein (CTEN/tensin 4), to influence focal adhesion signaling.

In parallel with their effects on IF solubility and mechanical properties, post-translational modifications can also modify the molecular interactions of IF proteins and thereby influence intracellular signaling. This has been nicely illustrated in the case of 14-3-3 proteins which specifically bind to phosphorylated keratin 8–18 IFs. The control of 14-3-3 localization by phosphorylation of keratins or vimentin strongly influences cell signaling. During S and G2 phase, keratins become hyperphosphorylated, which results in an increased soluble pool of keratin that sequesters 14-3-3 proteins away from the cell-cycle regulatory phosphatase Cdc25. Dephosphorylation of keratins releases 14-3-3 proteins as IFs return to their G1 filamentous state. Interestingly, recent observations suggest that the stable lamin network can dissociate from the nuclear lamina and become diffuse in the nucleoplasm depending on substrate rigidity. This observation opens exciting avenues for exploration. For example, the identification of the mechanism that regulates lamin organization may help us understand the consequences of such rearrangements in nuclear organization and possibly unveil new pathways controlling gene expression.

Combined with their intrinsic mechanical properties, the connection of the IF network with most cellular structures makes them excellent candidates to mediate mechanical cell responses, possibly tuning cellular functions, including gene regulation, by transmitting or protecting cells from both external and internal forces and mechanical strains.

Conclusions

The unique structural properties of IFs are at the core of their mechanical and signaling functions. As intracellular scaffolds, the cytoplasmic and nuclear IF networks integrate intracellular space, cell mechanics, and intracellular signaling to protect cells from physical, chemical or biological stresses. Cytoplasmic and nuclear IFs not only serve as guardians of intracellular organization and cell integrity, but also actively participate in signaling cascades to promote cell-cycle progression and cell survival or to inhibit apoptosis. Overall, IFs play an essential role in maintaining cell and tissue integrity during development and throughout the multiple physical, chemical or biological challenges encountered by living organisms. Most importantly, the dynamic control of IF protein expression and post-translational modifications modifies the mechanical and signaling properties of the IF network. This triggers cellular responses to environmental challenges, allowing cells and tissues to continuously adapt to their environment.

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Summary

Cells continuously adapt to their microenvironment. In particular, they modulate their morphology, growth, division, and motility according to the biochemical and physical properties of the extracellular matrix (ECM). Cells are equipped with adhesive structures called FAs, allowing them to interact with ECM proteins through the core transmembrane proteins called integrins and to sense the nature and the rigidity of the ECM. These information are transduced by FA proteins and lead, for instance, to changes in acto-myosin-mediated mechanical tension. Downstream signalling pathways also reach the nucleus; gene expression is then modified and may, in return, affect the composition of FAs or of the ECM proteins for adaptive cell response.

Here, we hypothesized that, in addition to signalling pathways, a direct mechanical coupling between the events occurring at the cell periphery and the nucleus may participate in the transmission of mechanical cues and the regulation of nuclear functions. Although intermediate filaments (IFs) have extremely interesting mechanical properties and resist high tension load, their involvement in mechanotransduction pathways remains elusive. Using astrocyte as a model, due to its specific combination of IFs: vimentin, GFAP, nestin, and synemin, we studied first the effect of substrate rigidity on the nucleus morphology and function, and on the organisation of IFs around the nucleus. Then, we investigated the role of IFs in rigidity-induced nuclear changes. Using a combination of microfabrication techniques, biochemical and microscopy methods, we showed that substrate rigidity affects the nucleus shape, volume, and structure of the chromatin and the recruitment of transcription factor (YAP) and IFs are mediating these changes. Our results suggest that IFs form a cage-like structure around the nucleus in a rigidity-dependent manner: stiffer substrates promote the formation of a cage of vimentin and nestin. In the absence of IFs, the nuclear changes induced by rigidity are different than with IF. The nucleus increases its size in soft substrate, together with an increase in tension measured by YAP localising in the nucleus. The structure of the chromatin is changed. Altogether, the results obtained during our investigation give a better understanding of the role of intermediate filaments in the mechanosensitive nuclear responses.