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Chapter 1

Glial Fibrillary Acidic Protein and Vimentin - cytoskeletal proteins at the crossroads of astrocyte cellular mechanics and signalling

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Chapter 1

The brain may well be the most complex system in the universe known to us. Every cell in the brain interacts with a multitude of highly specialized cells involved in either optimal neuronal communication or in the homeostasis of the central nervous system. Each cell has its own specific functions and properties. These individual cells, mainly neurons and glia, create an organized multicellular structure by interacting with and connecting to the extracellular matrix (ECM) and neighbouring cells, creating a structure of over 160 billion networking cells essential for all our conscious and unconscious brain functions (Azevedo et al., 2009). The cells reorganize the matrix and communicate with their direct and distant neighbours through various functional structures and signalling pathways to maintain a healthy organization and functioning of the brain. Much of this organization outside the cells is controlled by the configuration within the cell, provided by a great amount and diversity of proteins and peptides, each protein again with its own molecular structure and one or more functions.

One crucial structure in the organization of the cell is the cytoskeleton (Ingber, 1993). The cytoskeleton not only provides shape, motility, and structural scaffolding to the cell, but is also involved in signalling (outside-in and inside-out) (Jansen et al., 2015). The brain is an organ with highly specialized cell types and shapes, and correspondingly, brain cells contain a highly cell type-specific specialized cytoskeleton. The four main types of neural cells in the brain are the neurons and the several glia subtypes: the astrocytes, the oligodendrocytes, and the microglia. In general terms: neurons are electrically excitable cells highly specialized in rapid communication over long distances, astrocytes have important functions in brain homeostasis and neural communication (Sofroniew and Vinters, 2010), oligodendrocytes provide the brain with the fatty myelin to insulate the neurons (Bercury and Macklin, 2015), and microglia form the brain’s innate immune system (Hanisch and Kettenmann, 2007). These cell types derive from distinct progenitors (Zeisel et al., 2015), each with a developmental or functional cell state characterized by a specific and often unique cytoskeletal composition (Bauer et al., 2009; Fanarraga et al., 2009; Gitik et al., 2010; Lariviere and Julien, 2004; Mamber et al., 2013).

Cytoskeleton

Every cell’s cytoskeleton can consist of 4 distinct families of cytoskeletal proteins: actin filaments, microtubules, septins, and the intermediate filaments (IFs). Actin filaments and microtubules are ubiquitously present in all cells in the body and have been the subject of intense investigation over the last decades. Their role in cellular migration and intracellular transport makes them crucial components in
cellular survival and function (Gelfand and Bershadsky, 1991; Parsons et al., 2010). IFs have a thickness in between actin filaments (9 nm) and microtubules (25 nm), but were actually named IFs due to their 10 nm intermediate size in between thick (myosin) and thin (actin) filaments of skeletal muscle (Ishikawa et al., 1968). In 1976 glial filaments and neurofilaments were assigned “intermediate filaments” as their size was between microfilaments and microtubules (Yen et al., 1976). IFs were long considered to mainly have a role in the mechanical integrity of cells, but are now shown to have important signalling functions as well (Ivaska et al., 2007). Septins have recently been identified as a fourth cytoskeletal component. They have the ability to form ring- and filament-shaped structures required for organelles with specific membrane-shapes (Mostowy and Cossart, 2012).

IFs are a highly diverse group of proteins, comprising one of the largest protein families in vertebrates. This diversity in IF proteins is linked to cell type specific functions and architecture (Lowery et al., 2015). There are over 70 different mammalian IF members (see www.interfil.org), divided into 6 groups, based on structure and sequence homology. The nuclear Lamins (type V IFs) are represented by 2 genes in humans; Lamin A/C and Lamin B. The Lamins form the inside of the nuclear envelope, and accordingly it has been suggested that they originate from the oldest common eukaryotic ancestor, although it should be noted that plants do not have any IFs at all (Kollmar, 2015). Vertebrate cytoplasmic IFs are organized into the epithelial keratins (type I and II), non-Keratin self-assembling IFs (type III), neuronal self-assembling IFs (type IV), and non-self assembling and lens IFs (type VI) (Guérette et al., 2007). There are also classifications that assign Nestin and Synemin to the type IV category, and consider the lens IFs as a separate type due to the alternate assembly of these IF proteins into beaded filaments.

All of these different proteins are part of the IF family based on their related structure: they have a head domain, a rod domain, and a tail domain. The rod domain is very similar in the different classes of cytoskeletal IFs and can form a coiled coil through an IF-specific multimerization sequence (Herrmann et al., 2009). This structure allows the IF proteins to form a parallel dimer, that organizes into antiparallel tetramers, which will laterally assemble into the unit length filament (ULF) consisting of on average 32 monomers. These ULFs anneal longitudinally and compact to form the 10-nm filaments (Fig. 1). The antiparallel assembly step in the tetramer formation causes IFs to be unpolarized structures, making them unsuitable tracks for directed transport, this in contrast to Actin filaments and microtubules (Chernyatina et al., 2012; Strelkov et al., 2003).

Different tissues in the body have distinct IF expression profiles (Toivola et
Figure 1. Intermediate filament assembly and integration into the cytoskeleton

Top: The monomers of GFAPα and GFAPδ can assemble into a dimer, that can form a staggered antiparallel tetramer.

Bottom: IFs as a part of the complete cytoskeletal network that physically bridges the extracellular matrix (ECM) outside of the cell to the DNA in the nucleus of the cell. IFs are connected to the ECM through transmembrane Integrins (alpha and beta subunits), that form the anchorage points for focal adhesion complexes. These transmembrane complexes interact with IFs through Plectin, Plectin connects IFs to other cytoskeletal components (microtubules and actin), and ultimately Nesprin-3 connects IFs to the nuclear envelope. Nesprin-3 is a part of the trans-nuclear membrane LINC complex, binding the nuclear IFs, the Lamins. The Lamins subsequently play a role in the organization of heterochromatin and euchromatin, and are likely involved in regulation of transcription.
al., 2005). In neural cells of the central nervous system (CNS), the most common IFs are the Neurofilaments and α-Internexin in neurons and neural progenitors, and GFAP, Nestin, Synemin and Vimentin (Vim) in astrocytes and radial glia. IF proteins in microglia and mature oligodendrocytes, if expressed at all in vivo, typically do not form filaments that are distinguishable by electron microscopy or immunofluorescence, although few reports do describe the presence of Vim positive microglia/macrophages in pathology or in vitro (Graeber et al., 1988; Jiang et al., 2012; Kachar et al., 1986; Massa et al., 1984; Yamada et al., 1992). GFAP is alternatively spliced and 10 isoforms have been described (Kamphuis et al., 2012; Middeldorp and Hol, 2011). GFAPα is the canonical isoform. GFAPδ has an alternative tail domain (Fig. 2), and cannot form homodimers or networks by itself, but can incorporate into GFAPα or Vim networks (Nielsen and Jørgensen, 2004; Perng et al., 2008). GFAPδ levels are typically 1/10th of the GFAPα levels (Fig. 1).

The intermediate filament cytoskeleton of astrocytes

Astrocytes express four different IFs, Vim, GFAP, Synemin, and Nestin. Synemin and Nestin are type VI IFs that can not form filaments on their own, but are associated to the IF network (IFN). Although these two proteins are generally considered to be expressed in astrocytes, the expression levels of the proteins are highly heterogeneous in astrocytes throughout the brain, indicating a large molecular variation in astrocyte subtypes (Anderson et al., 2014; Oberheim et al., 2012). We will further focus on the other two, type III IFs. Vim and GFAPα are both able to form a network in a cell free system as well as in cells. Both Vim and GFAP have a distinct contribution to the ultrastructure in astrocytes, as the IFNs in Vim knockout (ko) and GFAPko astrocytes have a different ultrastructure best described as more compacted bundles with high density for VIMko, against lower contrast in electron microscopy, possibly more disperse filaments for GFAPko (Lepekhin et al., 2001; Menet et al., 2001).

Early functional studies on these filaments using either single GFAPko or double VIM-GFAPko mice revealed mild phenotypes (Liedtke et al., 1996; McCall et al., 1996; Pekny et al., 1995; Shibuki et al., 1996; Pekny et al., 1999). GFAPko mice showed enhanced neuronal long term potentiation in hippocampal neurons (McCall et al., 1996), and decreased long term depression in cerebellar parallel fiber-purkinje cell synaptic transmission, in addition to a reduced eye blink conditioning (Shibuki et al., 1996). Old GFAPko mice, over 12-18 months of age, are prone to white matter degeneration, poor white matter vascularization and variable increases in blood
Mutations in GFAP are the cause of Alexander’s Disease (AxD) (Prust et al., 2011). This disease is, in homology to many other IF protein-related or IF associated protein-related diseases, caused by gain of function mutations that induce aggregation. This is the case for lamin, keratin, neurofilaments and GFAP (Opal and Goldman, 2013; Russell et al., 2004; West et al., 2016). The mutations in GFAP result in a GFAP assembly defect and accumulation/aggregation in Rosenthal fibers in the brain, as well as in a cell free system (Perng et al., 2006). AxD is a neurological disease, and the most severe form can have an onset before the age of 4 years, with a severe loss of white matter. Rosenthal fibers can also be found in cases of severe gliosis or pilocytic astrocytoma, and have been described in a mouse overexpressing wild type (wt) human GFAP, indicating that high concentrations of wt GFAP can also induce aggregation (Hagemann et al., 2005; Wippold et al., 2006).

Vim is a widely expressed protein in cells of mesenchymal origin. Surprisingly, despite its wide expression, the initially generated VIMko mice displayed no obvious phenotype (Colucci-Guyon et al., 1994). Further investigations of the VIMko revealed quite diverging phenotypic alterations, ranging from impaired wound healing to decreased dilation of arteries (Eckes et al., 2000; Henrion et al., 1997). In addition, mutations in Vim can cause cataract in the lens (Song et al., 2009).

It is thought that much of the mild effects found in IF ko models are due to redundancy, as often multiple IFs are expressed in a single cell type. However, knocking out GFAP and Vim does not lead to compensatory expression of other IFs and does not lead to severe defects in astrocytes, despite the lack of IF networks as shown by EM. This raises the question: what is then the biological significance of IF proteins? Many data currently point towards IFs as central regulators in different kinds of cellular stress. This includes mechanical (Russell et al., 2004), toxic (Asghar et al., 2015), and hyperglycaemic stresses (Alam et al., 2013). For Vim and GFAP in astrocytes, this has been investigated by exposing the double ko models to a plethora of pathological stimuli and crossing the mice with different mouse models for brain diseases (Hol and Pekny, 2015). The absence of Vim and GFAP resulted in attenuated astrocytosis after injury. Functionally, this attenuated astrocytosis often results in aggravated progression of early injury, although at a later stage the lack of a gliotic scar formation presents the CNS with enhanced capability for regeneration (Hol and Pekny, 2015). However a recent, possibly paradigm-shifting study found that in a model of spinal cord injury, induced regeneration of axons was inhibited by two different methods of astrocytosis ablation, and posed that the glial scar is in fact required, but not sufficient for axonal regeneration, although the contribution of
the IFs in this model was not investigated (Anderson et al., 2016). It has to be noted that astrocytosis or reactive gliosis is a broad and vague term, and has a different meaning in different brain injuries/diseases (Pekny et al., 2016). In the rest of this introduction we will present what is currently known about the function of GFAP and Vim.

Molecular functions of Vim and GFAP

GFAP and Vim are both type III IFs. A wealth of knowledge is available for Vim, as this protein has been most studied. There are many research papers in which GFAP is used as a marker for astrocytes, but studies on the function of GFAP are scarce. We compared the amino acid sequence of GFAP and Vim to get an indication of complementarity or redundancy between these two proteins (Fig. 2). We observed, by using ClustalOmega, a mean sequence homology between human GFAP and Vim of 53%. The most conserved part of the IF proteins is the rod domain, due to the heptad repeat sequence required to form the coiled coil quaternary structure in the dimer stage of the filament formation (Chernyatina et al., 2012). The rod and tail domain have a sequence homology of 64% and 48%, respectively. The head domain only shows 23% homology. The rod has a strictly conserved length for the coiled-coil domains, but head and tail length varies across IFs, although Vim and GFAP have an equal tail length (GFAP (α and δ) head: 62 amino acids (aa), Vim head: 96 aa, Vim and GFAPα tails: 62 aa, GFAPδ tail: 61 aa). Between the other GFAP splice isoforms there are also different tail and even rod lengths (Hol and Pekny, 2015), whether there are variations in the head has not yet been confirmed. The head domain is essential for filament assembly in most IFs, as the heads of IFs (including GFAP) are typically predicted to prefer low solvent exposure, implying a buried state inside the filament (Kornreich et al., 2015). The tail domain is not always required for longitudinal assembly, depending on total IF protein composition in the filament, but the tail can regulate filament assembly (Chen and Liem, 1994). The tail of IFs is often depicted as protruding from the filament, but whether the tail is ordered or disordered in structure, and whether the tail associates to the rod or protrudes and can form crosslinks to nearby filaments is highly dependent on IF type and even specific protein within the IF family (Kornreich et al., 2015). GFAP and Vim are both predicted to have disordered tails with high solvent accessibility (Kornreich et al., 2015).

The tail of Vim is not essential for formation of filaments, but has a stabilizing role. A sequence conserved in the tail of type III IFs (KTXEXRDG) influences lateral
and longitudinal assembly, and can associate with the Vim rod (Kouklis et al., 1991; McCormick et al., 1993). For GFAP, the tail has been shown to be required for homodimer assembly, although the conserved RDG sequence in the tail is not essential (Chen and Liem, 1994). For GFAPδ, the alternative tail domain results in inhibition of assembly, curiously not assigned to the omission of the RDG sequence, but to the gain of tail residues T411 and I412 (Nielsen and Jørgensen, 2004). The high number of serine, threonine and tyrosine residues in the heads and tails are potential targets for kinases, and indeed numerous of these potential phosphorylation sites have been confirmed experimentally. These sites, in addition to other less described post-translational modifications, are important for IF dynamics and interactions with IF associated proteins (IFAPs) (Omary et al., 2006). The observed variability in head and tail domains indicates functional differences between GFAPα, GFAPδ, and Vim (Fig. 2).

To study the cellular and molecular functions of proteins in cell culture, a powerful tool often used is interfering with the protein itself, which can be done by pharmacological agents, by genetically altering the expression level of the protein, or by inducing mutations in the protein to study the role of specific or multiple amino acids. In contrast to Actin and microtubules, there are not yet any specific pharmacological agents available for IFs that have been thoroughly characterized. A compound called Withaferin A induces disruption of the IFN, but also affects other pathways in the cell (Grin et al., 2012). More work on this is in progress and several approaches are becoming available, as recently reviewed (Ridge et al., 2016). Genetic approaches with modified IF proteins with deletions, mutated amino acids, or even domain swaps have been important in determining the domains that are required for the assembly into ULFs and the domains that enable longitudinal annealing into filaments (Chen and Liem, 1994; Herrmann et al., 1996; McCormick et al., 1993). In addition, phosphomutants have been used to study the role of phosphorylation in IF function (Tanaka et al., 2015). As the GFAP and Vim IFs themselves do not have any enzymatic function, we propose they can have two main modes of action. The first mode of action is through the mechanical properties they provide when forming a network, whereas the second is through IFs interacting with other cellular components, such as organelles or proteins and thus modulating the function of these interacting partners.

**Mechanical properties of GFAP and Vim networks**

Initially IFs were mainly regarded as contributors to cellular integrity. This was based on the multicellular networks that Keratins form in skin, and the fragile
skin and blistering diseases associated with Keratin mutations (McLean et al., 1994). Indeed upon stretching of keratinocytes, a remarkable 2-fold extension of the cells was achieved without rupturing (Fudge et al., 2008). Complete ablation of Keratin filaments in mice results in extensive skin damage, assigned to desmosomal disorganization (Bär et al., 2014). This points towards two different roles in mechanical resilience: IF’s role in mechanical strength or intracellular integrity versus IF’s role in cellular adhesion. However, in these Keratin mutants effects on mitochondrial composition and activity were also found, again demonstrating that IF function is not limited to mechanical structure, and that diseases due to IF ablation or dysfunction can be due to mechanical as well as signalling changes (Kumar et al., 2015).

In the cell, IFNs can provide mechanical sturdiness, but single filaments also have remarkable mechanical properties. For three different IF proteins probed with atomic force microscopy (AFM) in vitro, it has been demonstrated that the filaments can be stretched on average 2.6 fold with concomitant thinning of the filament, implying structural changes in the filament (Kreplak et al., 2005). In addition to this astonishing mechanical resilience of the single filament, networks of IFs also display remarkable properties in cell free systems. Networks of Vim display stress-stiffening behaviour, increasing their elastic modulus over 100-fold when the network is stressed (Lin et al., 2010a). This makes the IFN highly resilient to deformation. Vim and GFAP also contribute to the mechanical properties of the cell, although in astrocytes they are typically less prominently exposed to mechanical deformation in comparison to the skin (Lu et al., 2010; Mendez et al., 2014). Vim contributes to the elasticity of mouse embryonic fibroblasts as demonstrated by a decrease in the shear modulus $G'$, a measure of mechanical stiffness, from 9 Pa to 4.5 Pa in VIMko fibroblasts as measured by active microrheology (Guo et al., 2013). This mechanical contribution to the cell contributes to the anchoring of organelles and the resistance of the cell to deformation. Induction of GFAP in astrocytes has been reported to have opposite mechanical consequences. In two studies, reactive astrocytes were generated, verified for GFAP increase, and subsequently mechanically tested by AFM. One study reported an increased stiffness, whereas the other reported a decreased stiffness of the astrocytes (Lu et al., 2010; Miller et al., 2009). This discrepancy could be attributed to the differences between the models, with an in vitro mechanical injury of isolated astrocytes in one experiment versus an intraocular pressure induced gliosis in Müller glial cells, followed by an isolation of the cells, in the other approach. In addition, astrocytosis is a complex process where more processes take place in the cell apart from GFAP and Vim upregulation (Pekny
et al., 2016). However, the expected mechanical effect of increased IF density would be an increased stiffness of the cells, based upon semiflexible polymer theory (Lin et al., 2010a). Depending on the IF protein and measurement method the stiffness of in vitro reconstituted IFNs increases with higher IF concentration, scaling with a power ranging from 0.5-2 to IF concentration, and resulting in elasticities of 1-10 Pa at low strains (Block et al., 2015; Lin et al., 2010a). In vivo this is more complex though, as composite networks consisting of multiple cytoskeletal proteins can behave differently depending on component interactions (Jensen et al., 2014).

GFAP networks can also be reconstituted in vitro from recombinant proteins (Perng et al., 2008). This approach has shown that the GFAP isoform GFAPδ induces a collapse of the IF network, but bulk rheological measurements on these in vitro networks have not yet been performed. Interestingly, Liedtke et al and Pekny et al reported softer consistencies of the CNS in the GFAPko and the VIM-GFAPko mice (Liedtke et al., 1996; Pekny et al., 1999).

Molecular interactions of GFAP and Vim in signalling

Driven by the identification of many diseases caused by IF mutations, the emergence of IF function in cell stress, and the correlation between IF expression and developmental state, many studies have proceeded to further clarify the role of IFs at a molecular level. This effort has generated a wealth of information on the biology of IFs, and showed the strikingly broad spectrum of processes that IFs play a role in. For Vim this spectrum extends, but is not limited to, regulation of cell adhesion, vesicle trafficking, migration, autophagy, and regulation of transcription factors, as extensively reviewed before (Ivaska et al., 2007).

Some of these processes have been mapped to specific interaction sites of Vim, which, when aligned to GFAP show practically no overlap (Fig. 2). Still, there is overlap between the functional processes GFAP and Vim are involved in. This will be further demonstrated per biological function in the following section.

Trafficking

Both Vim and GFAP are involved in trafficking of vesicles. A recent review on IFs in vesicle trafficking summarized the various described interactions with the Rab machinery, proteins that are responsible for regulating many steps in trafficking (Margiotta and Bucci, 2016). Direct mechanisms are emerging, as phosphorylation of the Vim head is required for recycling vesicle dependent integrin turnover, dependent on an interaction with PKCε (Ivaska et al., 2005). Another important pathway where Vim and/or GFAP were shown to have a role in, is the activation
mediated endocytosis of Jag1, a component of Notch signalling (Wilhelmsson et al., 2012). Whether this takes place through an interaction with Jag1 or (regulators of) the trafficking machinery, remains to be investigated. Altered trafficking increases differentiation of neural stem cells into neurons and increases engraftment in VIM-GFAPko (Widestrand et al., 2007; Wilhelmsson et al., 2012). Cocultures of neurons and astrocytes revealed that knocking out GFAP results in enhanced neuronal survival, related to increased ECM and adhesion protein expression (Menet et al., 2001). Astrocytes lacking GFAP and stimulated by ionomycin, ATP or interferon-γ, show a difference in the motility of vesicles, depending on vesicle subtype (Potokar et al., 2010; Vardjan et al., 2012). Interestingly, GFAP is enriched in gliosomes,
vesicles excreted by astrocytomas and also containing proteins from the exocytotic machinery. These vesicles play a role in glutamate transport (Milanese et al., 2009). Links for GFAP in receptor trafficking emerge from the accumulation of GLT1 in intracellular vesicles after dibutyryl cAMP treatment in GFAPko astrocytes, whereas in wt this induction results in localization of GLT1 to the membrane (Hughes et al., 2004). Due to the importance of vesicle release and trafficking in astrocytes, it is likely that the scope and importance of GFAP control over trafficking in astrocytes is much larger than so far established and may contribute to gliotransmitter release.

Vim and GFAP also play a role in autophagy, which is related to vesicle trafficking. Autophagy is induced in AxD by the accumulation of GFAP, and the proteasome is inhibited by the mutant form of GFAP (Tang et al., 2010). Mechanistically this may be due to an interaction between GFAP in oligomeric form and LAMP-2A, a lysosomal membrane protein that stabilizes chaperone mediated autophagy. Vim has been shown to interact with the vesicle adaptor complex AP-3. Both LAMP-2A and AP-3 are involved in the formation and sorting of autophagic vacuoles (Bandyopadhyay et al., 2010; Styers, 2004).

Motility

Vim and GFAP are both involved in cell migration and motility (Ivaska et al., 2007). In astrocytes from VIM-GFAPko mice, the loss of GFAP and Vim has an incremental effect on migration in vitro, meaning that the motility is further reduced with loss of each IF (Lepekhin et al., 2001). For Vim various mechanisms and interactions have been discovered that influence migration (Helfand et al., 2011), integrin trafficking (Ivaska et al., 2005), and on a more mechanistic level, migration related kinase modulation (Dave et al., 2013; Havel et al., 2015; Vuoriluoto et al., 2011). This effect on migration has been clearly demonstrated by the injection of Vim protein into the cell, which can induce lamellipod formation and migration (Helfand et al., 2011; Mendez et al., 2010).

The role of GFAP in migration is less straightforward. Loss of GFAP leads to increased invasiveness and reduced adhesion of astrocytoma cells (Rutka et al., 1999). This contrasts with the reported reduction of migration in GFAPko astrocytes (Lepekhin et al., 2001). The effects on migration are not only mediated by the interactions with the focal adhesions, but also by the polarization of cells and positioning of the nucleus (Dupin et al., 2011). Interestingly, the siRNA mediated knockdown of Vim and nestin alone resulted in a loss of cellular control on nuclear positioning, but GFAP by itself did not, although when GFAP is knocked down in the
context of Vim and nestin knockdown, the effect on nuclear positioning is enhanced (Dupin et al., 2011). The interactions GFAP has with the adhesion machinery are not known yet, although first indications for an effect on focal adhesion size have been described by us (Moeton et al., 2016).

**Cell-cell adhesion**

IFs are also important for cell-cell contacts. This is essential in keratinocytes where Keratins form a network structure spanning multiple cells at desmosomes and hemidesmosome formation. Vim also forms complexes with cell-cell interaction proteins. One of these adhesion proteins is VE-cadherin in endothelium, which is coupled to the IFN via desmoplakin (Vincent et al., 2004). Vim also co-localizes and probably interacts with α-catulin, a novel catenin like protein with roles in migration/adhesion (Bear et al., 2016). For GFAP no interactions have been described with the cell-cell adhesion machinery, but in the following chapters we describe transcriptional effects on proteins from these cell-cell complexes by GFAP modulation.

**Kinases targets/scaffolds**

GFAP and Vim, like all cytoplasmic IFs, are highly regulated by post-translational modification (PTM). Best understood are the phosphorylations, but also other modifications occur such as sumoylation, ubiquitination, acetylation, and glycosylation (Snider and Omary, 2014). Phosphorylation of Vim has been investigated quite extensively. The PTMs have effects on protein interactions as well as on assembly dynamics of Vim (Ivaska et al., 2007; Sihag et al., 2007). Phosphorylation of GFAP mainly affects dynamics and network organisation during mitosis (Yasui et al., 1998). Other PTMs of GFAP are the modifications of arginines into citrullines (Jin et al., 2013). Interestingly, a bioinformatics screen for sumoylation sites in GFAP using SUMOplot™ (abgent), revealed four unreported high-probability sumoylation target motifs in the rod. Two of these were localized in linker 1 and linker 1-2, regions in the rod where the coiled coil is interrupted (Fig. 2). None of these sites were identified in Vim.

IFs are not only a target for kinases, but they can also act as a scaffold for kinases. Also heat shock proteins can bind to IFs (Perng et al., 1999). These interactions illustrate another function of IFs, namely as protein scaffolds. Heat shock proteins such as αB-crystallin (CRYAB) can bind to the IFs and prevent aggregation of IFs, whereas CRYAB mutations can induce aggregation (Sanbe et al., 2004). Proteins that are not functionally required at all times but should be readily available may be sequestered on the filaments. GFAP binds 14-3-3γ at pSer8, and 14-3-3ε
immunoprecipitates Vim and GFAP (Satoh et al., 2006; Li et al., 2006). The interaction between Vim and 14-3-3ε was suggested to be a sequestering interaction, to prevent 14-3-3ε from binding to other targets (Tzivion et al., 2000). Vim is also required for the dimerization of receptor tyrosine phosphatase β (RPTPβ), which is required for insulin signalling and vascular smooth muscle cell migration (Shen et al., 2015). Various components of insulin signalling are also regulated by GFAP modulation in our experiments, possibly hinting towards a broader role for IFs in this pathway (Chapter 4 and Chapter 5).

Other functions

Vim and GFAP are also implicated in various other functions, e.g. Vim forms cages around aggresomes of accumulated misfolded proteins (Johnston et al., 1998) and captures aggregated proteins in a fishnet like manner (Ogrodnik et al., 2014). Vim can bind collagen mRNA and stabilize it (Challa and Stefanovic, 2011). GFAPδ can interact with a part of the γ-secretase complex, Presenilin 1 (Nielsen et al., 2002), and thereby could potentially affect downstream targets of γ-secretase processing and thus affect APP or Notch signalling.

IFs can interact with transcription factors, either to sequester them or to bring them in position for phosphorylation. Interactions have been found between Vim and Menin, GFAP and Menin, and between Vim and p53 (Lopez-Egido et al., 2002; Virtakoivu et al., 2015; Yang et al., 2005). In the case of Keratin 17, even a functional interaction with transcription factor Aire was found in the nucleus, making Keratin 17 a strong modulator of the cytokine profile of these cells (Hobbs et al., 2015). We also studied the effect on the cytokine profile induced by Alzheimer’s Disease in GFAPko astrocytes and microglia, but although quite some factors were differentially expressed, no major anti/proinflammatory profile switch was found (Kamphuis et al., 2015).

IFs not only play a mechanical role in the organization of organelles, but can also functionally interact with them. This is described above for vesicles, but also occurs with mitochondria (Chernoivanenko et al., 2015; Nekrasova et al., 2011). In activated immune cells, Vim can interact with the NLRP3 inflammasome as well as with mitochondria. Activated inflammasomes process pre-cytokines into their mature form, and the interaction with Vim and mitochondria is proposed to control reactive oxygen species levels required for activation of the inflammasome (dos Santos et al., 2015). In addition Vim modulates mitochondria membrane potential and motility, the latter possibly through an anchoring domain (Chernoivanenko et al., 2015; Nekrasova et al., 2011). For GFAP, no interactions have been described
so far with mitochondria, but in astrocytes recently a novel type of very small mitochondria was discovered in the fine processes in the cell periphery, either associated with GFAP or with Ezrin (Derouiche et al., 2015). There are also three case reports describing AxD patients with abnormal mitochondrial function: one with mutation GFAP R88C (and an additional independent mitochondrial mutation), another with GFAP N386I, and a case of a mitochondrial mutation phenotypically resembling AxD (Cáceres-Marzal et al., 2006; Gingold et al., 1999; Nobuhara et al., 2004; Schuelke et al., 1999).

**Oligomeric, filamentous, or cleaved Vim and GFAP**

Some of the interactions and functions described above have been attributed to the insoluble fully assembled filamentous networks, but oligomeric or cleaved fragments of the IF proteins have also been described to have relevant functions. The recycling of integrin vesicles was attributed to oligomeric Vim (Ivaska et al., 2005). In endothelial cells, Vim cleavage by calpains is an important step in creating a soluble pool for angiogenesis (Dave and Bayless, 2014). GFAP can be cleaved by caspases, such as Casp6. This cleavage leads to an N-terminal domain that has a dominant negative network collapse effect, and a C-terminal domain that forms small particles in vitro, but stays soluble in co-assembly with full length GFAP (Chen et al., 2013). Cleaved Vim can be used in neurons as ‘cargo wagons’ for carrying AKT to injured sites by binding both AKT and molecular motors (Perlson et al., 2005). IFs are also important targets of Caspases in apoptosis, and Caspase cleaved Vim fragments are pro-apoptotic (Byun et al., 2001).

**Ion-IF interactions**

GFAP and Vim also have various functional interactions with ions. Zinc was recently shown to be an important factor in Vim assembly through an interaction with C328, a residue important for oxidative stress response in the rod domain, which is conserved in GFAP (C294, Fig. 2) (Pérez-Sala et al., 2015). Divalent cations function as a crosslinker of IFNs, stiffening the network in a cell free system (Lin et al., 2010b). These examples further extend the range of interactions and functions IFs have, and how these can be modulated.

**Combining signalling and mechanics, a role in mechanotransduction?**

Soon after the discovery of IFs it was clear that they have an important mechanical role in cells. This is now further established by the remarkable mechanical properties of the networks and the filaments and the IF-related diseases that lead to mechanical defects. A 1980s review already proposed that IFs are integrators of cellular space
(Lazarides, 1980). Now in the last 15 years there has been an explosion of findings attributing a central role for IFs in signalling. A logical thought-step to make at this point is to consider whether these mechanical and signalling properties of IFs interact.

The process of translating mechanical cues into biochemical signals is called mechanotransduction. This process can be separated into three steps: mechanotransmission, mechanosensing, and mecanoresponse (Hoffman et al., 2011). Mechanotransmission describes the loading of the force upon structures that relay the force unto the mechanosensing protein. This step is mechanically straightforward and can be considered, very simplified, as a rope pulled on. Mechanosensing is the quintessential step of mechanotransduction, where a force upon a protein typically elicits a steric effect, such as unfolding. Examples include Notch receptor protein exposing an activating cleavage site after an applied mechanical force (Gordon et al., 2015), calcium channels Piezo1 and Piezo2 opening by a mechanical force (Coste et al., 2010), or fibronectin proteins unfolding and displaying complete hidden signalling domains under cell-exerted forces (Klotzsch et al., 2009). The mecanoresponse relates to the downstream cascade of events that are induced by the mechanical cue. This response can be at the molecular level, such as the growing of a focal adhesion under tension (Wolfenson et al., 2009), or at the tissue level, such as the thickening of an arterial wall under hypertension (Hahn and Schwartz, 2009).

A powerful method to find proteins that unfold under mechanical conditions, is cysteine shotgun labelling. This technique uses chemistry that labels cysteines, a residue that can be exposed in a protein that is unfolded under mechanical conditions, and identifies these proteins with mass spectrometry or fluorescence. This has identified spectrins, proteins with multiple buried cysteines, as proteins that unfold under mechanical stress (Johnson et al., 2007). In the same study, Vim was identified as a protein that is 50% more exposed when mesenchymal stem cells are treated with blebbistatin, a compound that inhibits non muscle myosin II, the main molecular motor responsible for cellular contractile forces. This is remarkable considering there is only one cysteine in Vim to be labelled. However this does not automatically imply that Vim filaments expose the cryptic site under cell-exerted tension by unfolding of secondary or tertiary protein structure, as depolymerisation of the filament (unfolding of quaternary structure) would also expose the cryptic cysteine (Johnson et al., 2007). Depolymerisation of the filament can also be a downstream mecanoresponse of a mechanosensitive Vim kinase that phosphorylates and solubilizes Vim. The remarkable elongation of IFs under single
filament stretching however does imply that some form of monomer-shifting can occur (Kreplak et al., 2005). Hypothetically, if this behaviour occurs in vivo, two proteins functionally interacting while associated on the IF scaffold may be pulled apart and can either be activated or inactivated.

A recent methodological paper on Vim also summarizes some of the findings of Vim on contractility measured by growing cells in collagen gels (Ridge et al., 2016). In brief, contractility was initially reported to be reduced in VIMko cells (Eckes et al., 2000), but later it was shown that this was only true at low cell density in the collagen gel. A higher contractility was achieved when VIMko cells were present at higher cell density in the gel (Murray et al., 2014). This effect was attributed to the dominance of cell/matrix over cell/cell contacts. Adding fibronectin to these gels equalized the contractility of the VIMko and the wt cells, further illustrating the complexity of cellular mechanics (Mendez et al., 2014). Similar experiments for GFAPko, VIMko, and VIM-GFAPko astrocytes showed that there was no difference in collagen gel compaction (Pekny et al., 1999). Direct probing of the intracellular forces by force spectrum microscopy, a microrheological technique recently developed, showed that Vim does not have a direct effect on intracellular contractility (Guo et al., 2014), but the effects of Vim on gel compaction can be due to other processes, e.g. through anisotropic effects, meaning forces are differently oriented, or cell-cell signalling dependent processes.

IFs are not only sensors but also targets of the mechanoresponse. The elasticity or pliability of the ECM is felt by cells that continuously probe their adhesion surface. The mechanical properties of the ECM have profound effects on cell differentiation (Engler et al., 2006; Keung et al., 2011; Trappmann et al., 2012). Vim responds to the stiffness of the ECM by shifting the ratio of soluble/insoluble Vim with the highest soluble fraction at the physiological stiffness of cells in tissue (Murray et al., 2014). In a study where LaminA/C was established as a mechanical rheostat, various proteins were screened for a correlation of their expression with the mechanical stiffness of the environment. A U251 xenograft injected into mouse brain (0.5kPa) showed lower GFAP and Vim protein expression than of a U251 xenograft in the flank (4 kPa) (Swift et al., 2013). Similarly, implantation of stiff electrodes in the brain induces gliosis at the interface of tissue and implant. Comparing soft (0.5 kPa) with stiff (30kPa) implants revealed reduced gliosis and reduced GFAP and Vim expression at the border of the softer implant (Moshayedi et al., 2014).

Lastly, IFs can also take part in mechanotransduction by integrating the cytoskeleton as a whole. Actin microtubules and IFs traditionally interact through plectins, and many other crosslinking proteins have been identified. Vim interacts
with and reciprocally organizes actin in lamellipods during migration (Jiu et al., 2015), microtubules link to Vim through APC to keep the IFN spread out (Sakamoto et al., 2013), and Vim and IF-connected plectins play a role in the stability of mechanosensing focal adhesions (Dmello et al., 2016; Gregor et al., 2013; Ivaska et al., 2007). IFs are proteins that bridge the focal adhesions all the way up to the nuclear DNA, as they also interact with the nuclear IFs, the Lamins, via Nesprins and the LINC complex (Linker of Nucleoskeleton and Cytoskeleton) (Fig. 1) (Starr and Fridolfsson, 2010). Lamins subsequently are essential in the organization of chromosomes (Guelen et al., 2008), but whether there is any inside-out or outside-in signalling between the nucleus and the cytoplasm through the IFN is not yet established.

**Conclusion**

Currently an astounding number of cellular processes has been identified that are related to the presence and structure of IFs. Still, a single IF protein has a limited number of binding sites, for interacting proteins, how is it possible that so many interactions were discovered for this protein class? A plausible explanation is the large amount of IF monomers in the cell and the dynamic nature of the network. One can hypothesize that in an IFN, a protein that both acts on IFs and binds to IFs, such as is the case for some kinases, can bind to one monomer in the filament and perform phosphorylations on the next monomer. The degrees of freedom of protein complexes that can be built upon IFs when tails and heads are both close to one another as well as staggered across the filament are substantial. The number of interactions increases further when heterotypic networks are formed of multiple IF proteins, as is the case in astrocytes, and when non-filamentous soluble IF proteins can expose new domains for interactions. The rod-structure-as-scaffold based functionality allows for immense flexibility in function. The organization of the cell and the high density of IF monomers in a filament allows for localized control of specific interactions and the large complexity of IF functions. The fact that these functions are not essential for survival of a cell or organism, does not relate to survival in a world full of challenges. IFs are called a crisis command center of the cell (Hol and Pekny, 2015), providing an organism with robustness in homeostasis and stress, one of the most important properties in biology for evolutionary success.

In the diseased brain, glia maintain a balance between containment of injury, the inflammatory response, and repair processes. This balance depends for an important part on the interaction between microglia and astrocytes. One of the hallmarks of the diseased brain is the increased expression of IFs in astrocytes. The
extended, dynamic, and extremely flexible IFN, such as in astrocytes, likely plays an important role in the astrocytes’ ability to maintain homeostasis. How IFs provide astrocytes with robustness under the stresses that astrocytes are exposed to is a question that will be further investigated in the following chapters.