Insights into intermediate filament regulation from development to ageing

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Summary

Intermediate filament (IF) proteins comprise a large family with more than 70 members. Initially, IFs were assumed to provide only structural reinforcement for the cell. However, IFs are now known to be dynamic structures that are involved in a wide range of cellular processes during all stages of life, from development to ageing, and during homeostasis and stress. This Commentary discusses some lesser-known functional and regulatory aspects of IFs. We specifically address the emerging roles of nestin in myogenesis and cancer cell migration, and examine exciting evidence on the regulation of nestin and lamin A by the notch signalling pathway, which could have repercussions for our understanding of the roles of IF proteins in development and ageing. In addition, we discuss the modulation of the post-translational modifications of neuronally expressed IFs and their protein–protein interactions, as well as IF glycosylation, which not only has a role in stress and ageing, but might also regulate IFs during development. Although many of these recent findings are still preliminary, they nevertheless open new doors to explore the functionality of the IF family of proteins.

Key words: Intermediate filament signalling, Post-translational modification, Phosphorylation, Glycosylation, *O*-linked *N*-acetylglucosamine (*O*-GlcNAc), Stress, Ageing, Nestin

Introduction

Intermediate filaments (IFs) were originally assumed to be static structures that provided reinforcement for the cell, but they have emerged as dynamic cytoskeletal structures that are involved in a wide range of cellular processes during all life stages, from development to ageing, and in processes involving both stress and homeostasis. IFs are expressed in all cells and tissue types and at all stages of life. Although diverse (Box 1), IF proteins have certain common highly conserved structural features, including a head domain (at the N-terminus), a central rod and a tail domain (at the C-terminus), and are characteristically regulated by a complex set of post-translational modifications (PTMs). Phosphorylation is the most common PTM in IF proteins and occurs primarily on the head and tail domains to regulate IF assembly, function and signalling interactions (Hyder et al., 2008; Omary et al., 2006; Pallari and Eriksson, 2006). One key function for IFs is to act as molecular scaffolds, thereby enabling the activation and deactivation of kinases and associated proteins, such as cyclin-dependent kinase 5 (Cdk5), in order to facilitate signal transduction (Helfand et al., 2005; Hyder et al., 2008; Ivaska et al., 2007; Kim and Coulombe, 2007; Omary et al., 2006). Such kinases often phosphorylate IF proteins and alter their filamentous organization, rendering them more (or less) soluble, depending upon the type of interaction, which can subsequently influence the function of that IF protein. There have been a number of recent reviews that cover many aspects of IF protein regulation and function, and, in particular, their role in signalling, disease and stress (Coulombe et al., 2009; Eriksson et al., 2009; Godsel et al., 2008; Goldfarb and Dalakas, 2009; Herrmann et al., 2009; Liem and Messing, 2009; Omary et al., 2009; Toivola et al., 2009; Worman et al., 2009). It is the aim of this Commentary to highlight some of the more enigmatic but, nevertheless, intriguing aspects of IF function.

First, we examine the regulation of IF proteins during development and ageing, and in cancer, focussing on the roles of nestin, lamin A and vimentin. In the second section, we discuss some of the regulators of IF proteins during stress, with a focus on the role of newly discovered PTMs, such as that of the prolyl isomerase Pin1 in neurofilament (NF) phosphorylation and that of glycosylation as an alternative regulator of IF dynamics and function. Finally, we examine the roles of splice variants of IF proteins in regulating the cellular stress response and protein–protein interactions.

IF proteins in development and regeneration

Nestin as a regulator of developing tissues

The IF protein nestin is expressed during early development; as differentiation of the cell proceeds, it is replaced by vimentin, desmin, NFs or glial fibrillary acidic protein (GFAP), depending on the mature cell type. Nestin was first identified as a neuroepithelial progenitor cell marker in the central nervous system (CNS) (Lendahl et al., 1990), but it is also expressed in myogenic precursors (Sejersen and Lendahl, 1993), differentiating testis (Fröjdman et al., 1997) and both the developing and adult kidney (Bertelli et al., 2007; Chen et al., 2006), among other tissues. Following injury, nestin expression is induced in the CNS (Frisen et al., 1995; Lin et al., 1995; Shibuya et al., 2002), muscle (Cízková et al., 2009; Vaittinen et al., 1999), optic nerve (Xue et al., 2006) and kidney (Zou et al., 2006). Despite the specific accumulation of nestin in developing and regenerating cells, very little is known about its functions and regulation. The latest research has now begun to elucidate the functions of nestin and to identify its regulatory partners, indicating that it might be an important protein that drives some processes that are essential for both development and cancer progression.

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Cdk5 is associated with nestin in myoblasts, neuronal precursor cells (Sahlgren et al., 2003) and human podocytes (Bertelli et al., 2007). Cdk5 phosphorylates nestin, and this phosphorylation regulates the organization of nestin filaments (Box 2) during myogenic differentiation (Sahlgren et al., 2003). Interestingly, the interaction between nestin and Cdk5 is not limited to a typical kinase–substrate relationship. In neuronal precursor cells, nestin forms a scaffold for Cdk5 and the Cdk5 activator p35, thereby sequestering them to the cytoplasm, which consequently inhibits the proapoptotic function of Cdk5 during oxidative stress (Sahlgren et al., 2006). In turn, it has recently been demonstrated that Cdk5 is targeted by protein kinase C-ζ (PKCζ) during myogenesis (de Thonel et al., 2010). PKCζ is essential for proper myoblast differentiation. It targets Cdk5 by phosphorylating p35 at a site that triggers the activation of calpains, which then cleave p35 into p25. The cleavage of p35 into p25 creates a more stable protein fragment, and this fragment is capable of sustained Cdk5 activation, consequently promoting nestin reorganization (Fig. 1, right-hand side). An earlier study, in cultured neurons, demonstrated that Cdk5 is also able to regulate p35 by phosphorylation, thereby protecting it from calpain-mediated cleavage (Kamei et al., 2007). Furthermore, a recent study has shown that nestin has the capacity to modulate Cdk5-mediated differentiation and terminal organization of muscle cells (Pallari et al., 2011) (Fig. 1, left-hand side). Taken together, these results suggest that a balance between Cdk5 and PKCζ is important for proper myogenic differentiation, as they show that disturbance of the PKCζ–Cdk5–nestin signalling pathway leads to nestin reorganization and impedes myoblast differentiation, implying that nestin also plays a key role in regulating myogenesis.

The interactions between nestin, Cdk5 and p35 during development and differentiation might also be important in cancer. Cdk5 and its activator p35 are expressed in the majority of prostate cancer samples and also in normal prostate tissue (Strock et al., 2006). Inhibition of Cdk5 activity substantially decreases the motility of prostate cancer cells and reduces metastasis (Strock et al., 2006), which results from an abnormal microtubule reorganization. However, that study does not mention any effects on microfilament or IF organization, which have also been shown to be important in cell motility (Helfand et al., 2003; Mitchison and Cramer, 1996).

It is hardly surprising that a role for nestin in cancer is emerging. Nestin expression is a marker of stem cells (Wiese et al., 2004), whose typical features of proliferation and migration are also important for cancer progression. Indeed, nestin is expressed in several cancers, such as glioma and colorectal cancer, as well as prostate cancer (Dell'Albani, 2008; Kleeberger et al., 2007; Teranishi et al., 2007). It has been suggested that nestin promotes the migration of prostate cancer cells and metastasis, as nestin knockdown decreases the motility of cells that have been derived from metastasized prostate cancers and the ability of metastatic rodent prostate cancer cells to metastasize following xenografting (Kleeberger et al., 2007). The underlying mechanism has not yet been elucidated, but we speculate that the functions of Cdk5 and nestin in prostate cancer migration might be functionally interlinked (in that Cdk5 regulates nestin organization, and nestin, in turn, regulates Cdk5 localization and activity). As mentioned above, nestin also has the capacity to act as a survival factor to inhibit Cdk5-mediated apoptosis (Sahlgren et al., 2006), which is crucially important during development and cancer progression.

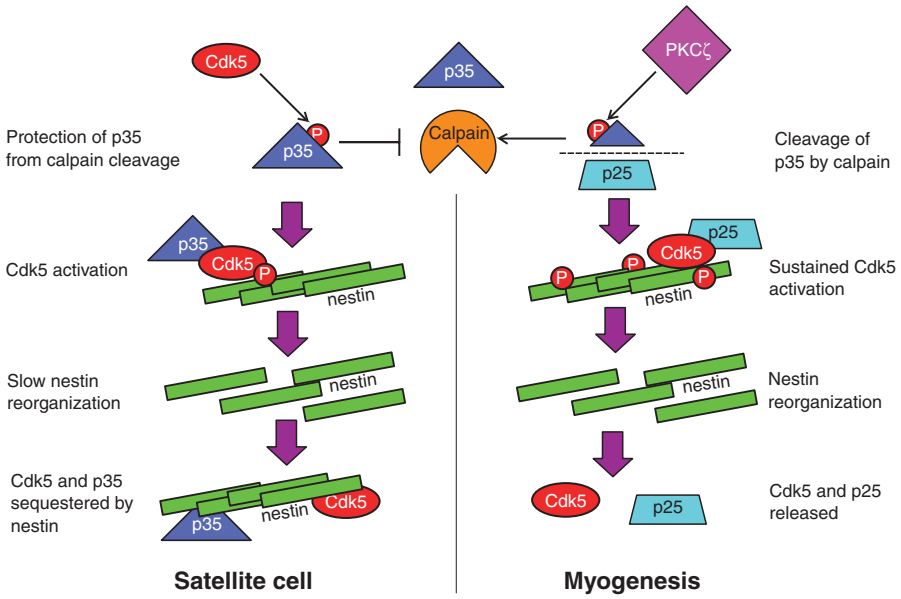

Fig. 1. Proposed model for the regulation of nestin through Cdk5 signalling during myogenesis and in undifferentiated satellite cells. The IF protein nestin can bind to and thus temporarily inactivate Cdk5 and Cdk5 activator p35 (Sahlgren et al., 2003; Sahlgren et al., 2006). In neurons, Cdk5 is able to phosphorylate p35, thereby protecting it from cleavage by calpain (Kamei et al., 2007); the same mechanism has been proposed to be active in undifferentiated muscle stem cells (satellite cells), as shown in the left-hand side of the figure (Pallari et al., 2011). When protected from calpain-induced cleavage, p35 can bind to and activate Cdk5, which in turn phosphorylates nestin, leading to its reorganization. In satellite cells, this process is, however, slow and most of the Cdk5 and p35 remain bound to nestin. During myogenesis, as shown in the right-hand side of the figure, phosphorylation of p35 by PKCζ overcomes the Cdk5-induced protection and promotes the cleavage of p35 cleavage into p25 by calpain. The p25 fragment is more stable than p35 and is able to bind to Cdk5 and promote its sustained activity, leading to nestin reorganization and subsequent release of bound Cdk5 and p25. If this step is impeded, myogenesis cannot proceed.

Notch and growth factor regulation of IF expression

The development- and tissue-specific expression pattern of nestin indicates that its expression, and its functions that are specific for given developmental stages and tissues, must be carefully controlled. Loss of nestin is an early sign of differentiation in neuronal stem cells and is triggered by the notch signalling pathway 'turning off' (Mellodew et al., 2004). The notch signalling pathway is essential for the regulation of cell fate and development, and is dysregulated in cancer (Allenspach et al., 2002; Dell'Albani, 2008; Harper et al., 2003; Jundt et al., 2008; Leong and Gao, 2008). Active notch signalling can promote and sustain nestin expression, through activation of the nestin gene promoter, in glioblastoma multiforme (Shih and Holland, 2006). As nestin is a marker of stem cells, and notch signalling is highly active during the process of cell fate determination, it has been suggested that control of nestin expression by notch signalling could contribute to glial tumour development by maintaining tumour cells in a more undifferentiated progenitor state (Shih and Holland, 2006). Acinar-to-ductal metaplasia, in which pancreatic cells transdifferentiate, also requires notch signalling. Notch activation in response to epidermal growth factor (EGF) signalling also leads to upregulated nestin expression and the accumulation of cells at an intermediate stage of metaplasia (Miyamoto et al., 2003). Considering that notch can directly affect nestin expression (Shih and Holland, 2006), it is possible that, in the case of metaplasia, the upregulation of nestin expression is a direct response to notch signalling and that notch is a positive regulator of nestin during development and tumorigenesis. Moreover, inhibition of notch leads to downregulation of Cdk5 activity (Kanungo et al., 2008). As described above, Cdk5 is a potent regulator of nestin organization, with nestin also acting as a scaffold for Cdk5 sequestration. It has also been shown that a decrease in Cdk5 activity leads to the inhibition of cortical development (Gilmore et al., 1998; Ohshima et al., 1996) and myogenesis (Lazaro et al., 1997; Philpott et al., 1997). It is therefore tempting to speculate that because notch can regulate Cdk5 activity, notch signalling affects not only nestin expression but also Cdk5-mediated nestin reorganization during neuronal and muscle development. Again, such a notch-orchestrated interaction between nestin and Cdk5 could regulate the survival of differentiating or metastatic cells (Sahlgren et al., 2006).

Whereas nestin expression is induced by notch, lamin A, a splice variant of the lamin family (which are the nuclear IFs), is able to activate the notch signalling pathway. The lamins are strongly associated with the premature ageing disease Hutchinson–Gilford progeria syndrome (HGPS) (Cao and Hegele, 2003; De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003), which is characterized by extremely early onset of ageing (Ding and Shen, 2008; Hennekam, 2006). HGPS (among other so-called laminopathies) is caused by mutations in the gene encoding lamin A, some of which disrupt the assembly of lamin A filaments and cause defects in nuclear architecture and chromatin organization (Taimen et al., 2009). The most common form of HGPS results from a lamin A mutation that leads to incorrect splicing of lamin A to form progerin, which cannot assemble into mature lamin A filaments (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003). Progerin is expressed at low levels in healthy individuals and participates in the normal ageing process (Cao et al., 2007; McClintock et al., 2007; Scaffidi and Misteli, 2006). It is poorly understood how the inability to form mature lamin A translates into the overwhelming symptoms experienced by HGPS sufferers, including growth retardation, loss of subcutaneous fat and low bone density (Sarkar and Shinton, 2001), but there is accumulating evidence that key signalling pathways involved in development might be responsible. HGPS occurs in cells of mesenchymal origin (Csoka et al., 2004), whose differentiation is affected by progerin. Progerin expression has been shown to induce activation of downstream components of the notch signalling pathway and the subsequent expression of differentiation markers, such as nestin, in undifferentiated cells, implying that high levels of progerin accelerate the normal differentiation process (Scaffidi and Misteli, 2008). However, during the normal ageing process, the low levels of progerin could also contribute to ageing by affecting mesenchymal stem cell differentiation in a notch-dependent manner, leading to aberrations in tissue homeostasis that are consistent with the hallmarks of ageing (Scaffidi and Misteli, 2008). From these studies, it is clear that notch regulates IF expression and function, and that notch signalling is also regulated by IFs.

Nestin expression is also induced in muscle and neuronal tissues following injury (About et al., 2000; Frisen et al., 1995; Lin et al., 1995; Vaittinen et al., 2001). The mechanism by which nestin expression is stimulated in this process remains largely unclear; however, it has recently been shown that nestin expression can be induced through the EGF signalling pathway by extracellular signalling molecules, such as EGF and thrombin, in vascular smooth muscle cells (Huang et al., 2008; Huang et al., 2009). In addition, transforming growth factor β1 (TGFβ1) is also required for the re-expression of nestin in astrocytes with multiple sclerosis lesions (Moreels et al., 2008). The effect of extracellular signalling molecules on nestin expression is dependent on the cell type and differentiation state, as shown by a study examining the effect of thrombin on nestin expression in various cells (Wautier et al., 2007). In that study, thrombin was found to enhance expression of nestin in radial glial cells, whereas it decreased nestin expression in mesenchymal stem cells. Thus, the regulation of nestin is clearly complex, and the picture that emerges is that nestin expression in development is regulated in part by growth factor signalling. Although EGF is the only growth factor that is known to regulate nestin expression, it is not far-fetched to consider that nestin expression could also be regulated by other growth factors, such as Wnt and TGFβ, and other developmental pathways, depending upon the developmental stage and cell type.

Remodelling of IF proteins in neurons

Studying the role of the IFs during neurodegeneration has yielded much of our current knowledge of IF function in the CNS. However, the role of IFs in the developing CNS has been less studied. IFs are differentially expressed during neuronal development; however, the type IV IFs (Box 1) are dispensable as none of the mouse knockout models generated for type IV IFs (Box 2) displays gross developmental defects (Elder et al., 1998; Levavasseur et al., 1999; McCullagh et al., 2008; Rao et al., 1998; Zhu et al., 1997). NFs are known to affect the development of a subset of neuronal cell types (for a review, see Lariviere and Julien, 2004). Briefly, NFs regulate the radial growth of axons, which in turn affects conduction velocity (Perrot et al., 2008) and are required for proper dendritic arborization (i.e. the branching of dendrites) of large motor neurons (Kong et al., 1998). Much of the work aimed at dissecting the functions of NFs in the CNS has concentrated on NF phosphorylation and its role in neuronal transport (for reviews, see Barry et al., 2007; Shea et al., 2009; Sihag et al., 2007). Less understood is how extracellular cues, for example from neurotransmitters, affect NF function and regulation

in the developing brain. Glutamate, an abundant neurotransmitter, interacts with *N*-methyl-D-aspartate (NMDA) receptors. These are the primary receptors that control synapse plasticity (i.e. the ability of the connection between two neurons to change in strength) and memory function. Blocking of NMDA receptors, with an antagonist, increases the neurite outgrowth of developing cortical neurons and the phosphorylation of NF-medium (NF-M) (Box 1), which increases its solubility (Fiumelli et al., 2008). The authors of that study speculate that NF reorganization plays a role in regulating neuronal outgrowth but do not propose a mechanism. One clue to the underlying mechanism comes from studies showing that glutamate excitotoxicity contributes to hyperphosphorylation of NF, leading to NF aggregation and to disturbed NF transport in developing neurons. This process is mediated through modulation of the c-jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) pathways (Ackerley et al., 2000; Asahara et al., 1999; Zamoner et al., 2008). The stress-associated protein kinase JNK is present at basal levels in developing neurons and also has non-stress-related functions (Gelderblom et al., 2004). Thus, one could speculate that glutamate signalling in the developing brain stimulates the JNK (and ERK) pathway, which in turn would regulate normal NF-M phosphorylation and transport, thus promoting neurite outgrowth in the developing neuron.

Another important study that investigated IF signalling, in a neuronal model system, demonstrates a role for vimentin in neuronal regeneration (Perlson et al., 2005). Vimentin is expressed in early neuronal precursors and during neuronal regeneration. It was shown that soluble vimentin, which in this case results from local translation and calpain cleavage, can interact with an importin–dynein complex and phosphorylated ERK following nerve injury. The resulting vimentin–importin–dynein–(phosphorylated ERK) complex moves along microtubules, in a retrograde manner, from the site of the lesion in the axon until it reaches the ganglia, where phosphorylated ERK dissociates from the complex. The interaction of phosphorylated ERK with vimentin was shown to protect ERK from dephosphorylation, thus maintaining its activation and signalling (Kholodenko, 2002) in a Ca^{2+}-dependent manner (Perlson et al., 2005; Perlson et al., 2006). This interaction between phosphorylated ERK and vimentin has a positive effect on neuronal outgrowth, indicating that vimentin is important for the regeneration of neurons following injury (Perlson et al., 2005). Such a function of vimentin as a molecular scaffold might also occur during neuronal development, in order to enable efficient signal transduction along the length of the growing neuron. The function of soluble IFs has largely not been studied, perhaps because they have primarily been perceived as by-products of IF phosphorylation and reassembly. However, the work described above clearly demonstrates that soluble IF proteins can act as scaffolds, independently of insoluble IF filaments.

Despite the advances, discussed above, with regard to the roles of IF proteins in the development of multicellular organisms, this aspect of the IF field is still very much in its infancy. This is mainly because it is difficult to establish model systems to study IF functions in development and regeneration; as the IF gene family is so multifaceted, tampering with one specific family member, for example by knockdown, can be compensated for by another IF protein. Furthermore, working with IF proteins is challenging; although they are relatively insoluble, they, nevertheless, exert strikingly dynamic behaviour. As techniques and our experience with model systems improve, we believe that this area of IF research could not only prove to be highly exciting but is also likely to enhance our understanding of the mechanisms underlying tissue development.

IF regulation during neuronal stress and ageing

Throughout their lifetime, cells are subjected to many stresses, such as osmotic, oxidative, UV, thermal, mechanical, chemical and metabolic stress. In response to stress, the cell will upregulate a number of proteins, including molecular chaperones, which act to prevent protein denaturation and enable appropriate protein refolding, and activate stress response signalling pathways, such as the JNK pathway. In some cases, misregulation of IF assembly or their PTMs, and the subsequent aggregation, can trigger the stress response; however, the induction of a stress response can conversely trigger IF protein aggregation. Prolonged or increased cellular stress responses often lead to disease, such as Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS), and have also been implicated in ageing. As mentioned above, there are a number of reviews that cover this subject in more detail; the section below is thus intended to highlight some specific and important aspects of IF regulation and functions during stress-related disease and ageing.

Box 1. IF protein subtypes

(for reviews, see Herrmann and Aebi, 2000; Osborn and Weber, 1986; Oshima, 2007)

Type I and II: keratins

Type I and type II IFs are acid and basic keratins, respectively. Keratins are typically expressed in epithelial tissues and form heteropolymers comprising type I and II molecules. Perturbations in keratin expression and mutations in keratin proteins have been identified as causative factors in many skin and liver diseases (Ku et al., 2007; Uitto et al., 2007), emphasizing their indispensability for maintaining appropriate tissue function and integrity.

Type III: vimentin, desmin, GFAP and peripherin

Vimentin is the most widely expressed type III IF protein and derives from cells of mesenchymal origin, in contrast to desmin, which is expressed exclusively in muscle. Alterations in desmin expression and mutations in its gene have been associated with cardiomyopathies (Capetanaki, 2000; Hyder et al., 2008; Pallari and Eriksson, 2006). GFAP and peripherin are crucial for functions of the nervous system (Barclay et al., 2010; Messing and Brenner, 2003).

Type IV: neurofilaments, nestin, α-internexin, synemin and syncoilin

These IF proteins are mainly expressed in the nervous system. Neurofilaments are subdivided into heavy (NF-H), medium (NF-M) and light (NF-L). Some neurodegenerative diseases result from abnormal accumulation of type IV IF proteins (Barry et al., 2007).

Type V: lamins

Lamins are considered non-filamentous IFs, as they form a mesh network surrounding the nucleus. Mutations in lamins cause a wide variety of disorders, the so-called laminopathies, which are distinctive in their aetiologies (Worman and Bonne, 2007).

Type VI: the lens IF proteins filensin and phakinin

These are the principal cytoskeletal element of the eye lens and are essential in maintaining lens cell architecture and the lens optical properties (Song et al., 2009).

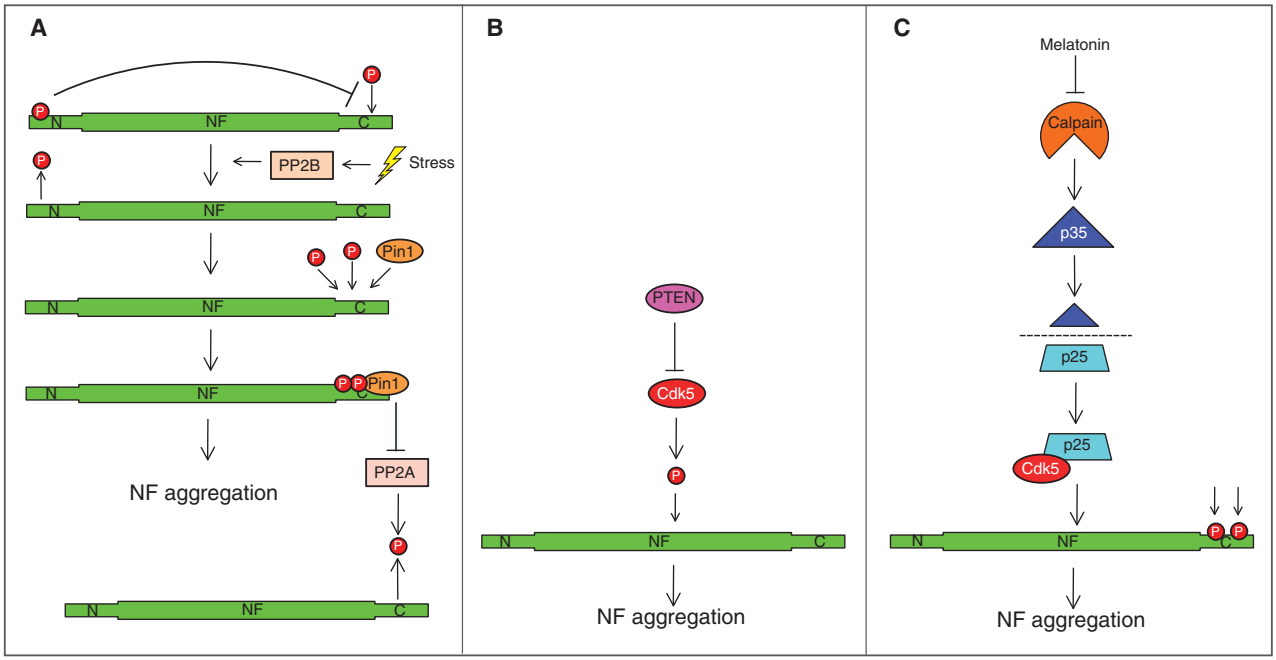

Fig. 2. Regulation of NF phosphorylation during stress and neurodegeneration. (**A**) In normal conditions, N-terminal phosphorylation of NFs inhibits the phosphorylation of the NF C-terminus. However, during stress, calcineurin [also known as protein phosphatase 2B (PP2B)] dephosphorylates the N-terminus, leaving the C-terminus open to phosphorylation and binding of Pin1 (Kesavapany et al., 2007). Pin1 also inhibits PP2A from dephosphorylating the C-terminus, leading to the hyperphosphorylation of NFs and their subsequent aggregation. (**B**) The role of Cdk5 in NF regulation. PTEN inhibits Cdk5 activity. In the absence of PTEN inhibition, Cdk5 hyperphosphorylates NFs and causes the aggregation of NFs. NF aggregates and loss of PTEN have been implicated in the pathology of AD (Griffin et al., 2005). (**C**) Regulation of Cdk5-mediated NF hyperphosphorylation through melatonin. We propose that melatonin could regulate Cdk5 activation by inhibiting calpain activity (Alvira et al., 2006), with the opposite effect to the upregulation of Cdk5 activity by PKCζ in myogenesis (Fig. 1). Calpains cleave the Cdk5 activator p35 into p25, which is capable of sustained activation of Cdk5, and, consequently, could contribute to NF hyperphosphorylation. As melatonin levels are decreased during ageing and also in AD (Liu et al., 1999; Wu et al., 2003), melatonin is no longer able to inhibit calpain and, thereby, prevent Cdk5 activation and the subsequent NF phosphorylation and aggregation.

Protection against NF hyperphosphorylation during stress

Hyperphosphorylation of NFs is characteristic for the pathological accumulation of NFs in neurodegenerative diseases (Al-Chalabi and Miller, 2003). Typically, hyperphosphorylation occurs as a result of the misregulation of the kinases and phosphatases that are responsible for NF phosphorylation (for reviews, see Perrot et al., 2008; Rudrabhatla and Pant, 2010; Shea and Chan, 2008). Decreased levels of melatonin, the hormone important for maintaining circadian rhythm, are thought to be an early marker for AD (Wu et al., 2003), which is a debilitating neurological disease typified by loss of memory and cognitive impairment (Kelley and Petersen, 2007). During ageing, melatonin levels are typically reduced, and they are even lower in AD patients (Liu et al., 1999; Wu et al., 2003). It remains to be established, however, whether low melatonin levels are a cause or an effect of the disease. Misregulation of Cdk5, one of the kinases targeting NFs, is considered to be important in the pathology of AD (Crews and Masliah, 2010). Melatonin has been shown to modulate Cdk5 activity by preventing calpain-mediated cleavage of the Cdk5 activator p35 into p25, which mediates sustained Cdk5 activation (Alvira et al., 2006). This result suggests that melatonin might protect against aberrant NF phosphorylation (Fig. 2). The activity of Cdk5 and of the Cdk5 activator p35 and p25 proteins is regulated by the tumour suppressor phosphatase and tensin homologue (PTEN) (Nayeem et al., 2007), which also controls the phosphorylation of microtubule-associated protein tau, which when present in high levels is a marker for AD (Hampel et al., 2010). PTEN-deficient mice that show signs of neurodegeneration have elevated levels of activated Cdk5 and ERK1/2. Moreover, levels of phosphorylated NF-M, a Cdk5 and ERK1/2 target, are also increased in these mice, implying that, under normal physiological conditions, PTEN might prevent the hyperphosphorylation of NFs (Fig. 2) and their subsequent accumulation in Purkinje cells, which is a hallmark of some neurodegenerative diseases (Nayeem et al., 2007). It is tempting to infer from the above studies that there is functional interplay between the modulation of Cdk5 activity by PTEN and melatonin and the resultant phosphorylation of NFs. However, it is probable that melatonin and PTEN regulate Cdk5 independently of each other, as melatonin itself does not affect PTEN levels, although this has not been specifically confirmed in a neuronal model (Tam et al., 2007).

Pin1 as a modulator of NF phosphorylation and aggregation during stress

Abnormal phosphorylation of NFs, as described above, is not only caused by inappropriate kinase activation but can also be caused by events following phosphorylation. An example of such post-phosphorylation processing is through the ‘prolyl-isomerase cis-trans isomerase NIMA-interacting 1’ (Pin1) protein, which selectively regulates phosphorylated serine/threonine-proline motifs by cis and trans isomerization. This isomerization can affect the structural conformation of a protein, as well as its stability and, sometimes, its function (Wulf et al., 2005). NFs are phosphorylated on lysine-serine-proline (KSP) motifs, and up to 100 of these motifs are present in the NF tail domain (Rudrabhatla and Pant,

Box 2. IF assembly and general function

IF protein assembly

The cytoskeleton consists of microtubules, IFs and microfilaments. IFs are assembled when hetero- or homo-dimers of IF proteins form tetramers, which then associate into unit-length filaments (ULFs) comprising eight tetramers. ULFs can anneal longitudinally to form radially loosely packed filaments. A radial compaction step causes rearrangement of ULF structures and reduces their widths to ~10 nm, thus producing mature filaments (Godsel et al., 2008). Keratins assemble by forming heteropolymers comprising type I keratins and type II keratins (Coulombe, 1993). The NF subtypes NF-H, NF-M and NF-L, and possibly also α-internexin, assemble into filaments with each other. Other type IV IFs assemble with other types of IFs (Barry et al., 2007). Assembly and disassembly of IFs is regulated by PTMs, as outlined in the main text.

Concepts of IF function

In contrast to actin and microtubules, IFs are flexible and elastic and can thus withstand large deformations without breaking and are able to absorb mechanical stress (Lazarides, 1980; Wagner et al., 2007). In addition, IFs can regulate cell signalling, for instance keratin 8 has been observed to act as a 'phosphate sponge'. This model suggests that stress-activated kinases phosphorylate sites on IFs instead of those on other substrates, thus delaying the propagation of kinase signalling (Ku and Omary, 2006).

2010). NFs therefore constitute Pin1 targets. Downregulation of Pin1 in glutamate-stressed cells, a model of the motor neuron disease ALS, leads to decreased levels of phosphorylated NF-heavy (NF-H) and prevents its aggregation in neuronal perikarya, as well as inhibiting glutamate-induced apoptosis (Kesavapany et al., 2007). This suggests that Pin1 activity could promote the aggregation of NF-H and the progression of ALS. Pin1 has been shown to regulate NF-H phosphorylation through the proline-directed kinases ERK1/2, Cdk5 and its activator p35, and JNK3. During oxidative stress, one of the characteristic features of AD, NF-H is hyperphosphorylated by ERK1/2 and JNK, and Pin1 abrogation can rescue the effects of this stress-induced hyperphosphorylation (Rudrabhatla et al., 2008). Furthermore, Pin1 can contribute to NF hyperphosphorylation by promoting isomerization of the phosphorylated NF protein, which prevents protein phosphatase 2A (PP2A)-mediated NF dephosphorylation independently of the ERK, JNK and Cdk5 pathways (Rudrabhatla et al., 2009). These results concur with the aforementioned studies and, taken together, suggest that Pin1 activity in neurodegenerative disease stimulates NF hyperphosphorylation.

Maintaining the correct topographical phosphorylation of NF-H is essential for axonal transport in a neuron. It has been proposed that, during stress, the ratio between kinases and phosphatases is tipped in favour of proline-directed kinases, such as ERK1/2, JNK and Cdk5. Under normal conditions, phosphorylation of the N-terminal NF head domain prevents the phosphorylation of the C-terminus. However, during stress, calcineurin might dephosphorylate the head domain, which in turn would affect NF polymerization. Dephosphorylation of the N-terminus would also open up the C-terminus for phosphorylation and Pin1 binding. Essentially, Pin1 functions in stabilizing NF phosphorylation by inhibiting PP2A-mediated dephosphorylation of the C-terminus, thereby leading to NF hyperphosphorylation, with subsequent accumulation of NF into perikaryal aggregates (Fig. 2). Under normal conditions, Pin1 might not be able to interact with NF-H, owing to its C-terminal binding sites being inaccessible as a result of N-terminal phosphorylation (Kesavapany et al., 2007; Rudrabhatla et al., 2009). However, under stress and disease conditions, Pin1 can access its target sequences on NF-H, and thus facilitate NF-H hyperphosphorylation and the subsequent accumulation of abnormally phosphorylated cytoskeletal elements and hyperphosphorylated protein aggregates, with dire consequences for the neurons involved.

Glycosylation – an additional level of IF regulation

O-linked glycosylation (*O*-GlcNAcylation) is a crucial regulator of many cellular functions, such as mitosis, the stress response, nutrient sensing and signal transduction (Love and Hanover, 2005; Zachara and Hart, 2004; Zachara and Hart, 2006), and also contributes to the progression of diabetes (Copeland et al., 2008; Dias and Hart, 2007) and neurodegenerative diseases (Dias and Hart, 2007). Glycosylation is emerging as an additional signalling pathway that controls both the functions of IF proteins and their interactions. Glycosylation involves the modification of asparagine (*N*-linked), or serine and threonine (*O*-linked) residues. *O*-linked glycosylation usually involves the addition of an oligosaccharide but can also involve the addition of a single sugar [e.g. *N*-acetylglucosamine (GlcNAc) or mannose] – a single GlcNAc in the case of IFs. The reaction is catalysed by *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA), which add and remove the sugar moiety, respectively (Fig. 3) (Hart et al., 2007; Wells and Hart, 2003). *O*-linked glycosylation is as dynamic as phosphorylation, and both modifications frequently occur on the same or adjacent residues (Hart et al., 2007). This dynamic mutual relationship between *O*-GlcNAcylation and phosphorylation has been described as the 'yin-yang' hypothesis and refers to their alternative competing and complementary functions (Comer and Hart, 2000; Hart et al., 1995; Wells et al., 2004). Proteins can be concurrently phosphorylated and *O*-GlcNAcylated, and these PTMs can in turn regulate each other in a competitive manner and, moreover, also regulate other PTMs. Keratins, vimentin, nestin, GFAP, peripherin and NFs are all glycosylation targets (Chou et al., 1992; Dong et al., 1993; Grigelioniene et al., 1996; King and Hounsell, 1989; Korolainen et al., 2005; Slawson et al., 2008; Wrigley et al., 2002). Although the physiological functions of IF phosphorylation are relatively well established, as mutations of phosphorylation sites can affect filament organization and have pathological effects in cells or animal models, the physiological roles of glycosylation have remained enigmatic. However, new evidence suggests that IF glycosylation can have protective functions for the cell against injury and stress (Ku et al., 2010), and glycosylation also regulates IF solubility, protein stability and degradation (Srikanth et al., 2010).

Flexible functions of NF glycosylation during glucose deprivation

During glucose deprivation, a consequence of ischaemia that can lead to axonal damage, NF-H is *O*-GlcNAcylated, which increases its solubility. Solubilization of NFs can lead to a reduction of axonal diameter and an abnormal structure, which has a negative effect on conduction velocity (Perrot et al., 2008). The interaction between NF-H and OGT is not dependent on p38α, a member of the mitogen-activated protein kinase (MAPK) family of proteins known to phosphorylate NFs (Ackerley et al., 2004). However,

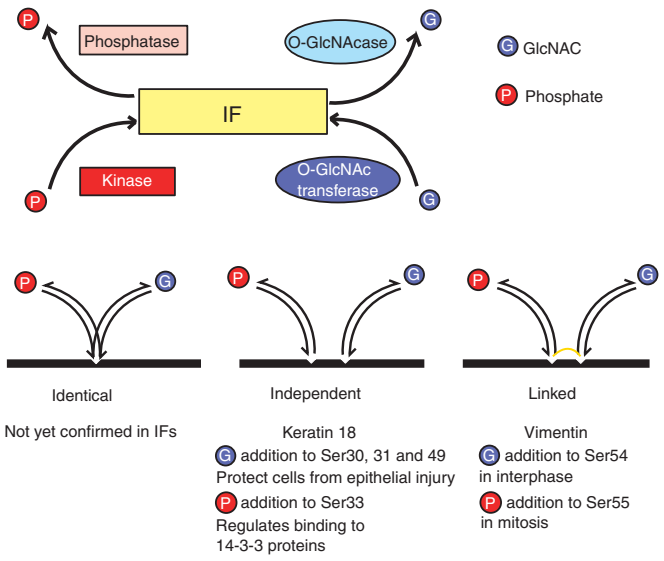

Fig. 3. Glycosylation and phosphorylation of intermediate filaments. Glycosylation can be either *N*-linked or *O*-linked. The type of *O*-linked glycosylation discussed here involves the addition of an *O*-*N*-acetylglucosamine (*O*-GlcNAc) onto a serine or tyrosine residue, which is performed by *O*-GlcNAc transferase. In the reverse reaction, an *O*-GlcNAcase removes the *O*-GlcNAc from the protein. As phosphorylation and glycosylation occur primarily on serine residues in IF proteins, both modifications can take place on the same residues and therefore compete with each other, as shown in the lower-left of the figure. Although this has not been confirmed in IFs to our knowledge, it has been shown for other proteins (Hart et al., 2007). Glycosylation and phosphorylation can also occur on adjacent residues. This can occur independently, as in keratin 18, which has glycosylation sites at serine 30, serine 31 and serine 49, which either together or alone protect epithelial tissue from injury, and a phosphorylation site at serine 33, which regulates binding to 14-3-3 proteins (Ku et al., 1998; Ku et al., 2010). Adjacent residues can also affect each other, as is the case for vimentin where glycosylation of serine 54 is evident in interphase cells and phosphorylation of serine 55 occurs in mitosis (Slawson et al., 2008; Wang et al., 2007). These variations add additional complexity to subsequent signalling events (Comer and Hart, 2000; Hyder et al., 2008).

p38α activation is required to mediate the recruitment of OGT to NF-H and, therefore, to increase its *O*-GlcNAcylation in response to glucose deprivation (Cheung and Hart, 2008). NF-M can be reciprocally O-GlcNAcylated and phosphorylated during glucose deprivation, as well as in AD (Deng et al., 2008). The authors of that study found that, in fasting rats, a decrease in glucose uptake or metabolism leads to downregulation of NF-M *O*-GlcNAcylation and, consequently, an increase in phosphorylation. This type of reciprocal regulation has been postulated to act as a ‘metabolic switch’. Although the aforementioned studies appear to contradict each other, they examine glucose deprivation by different methods and focus on different NF types. The apparent discrepancy could thus be explained by differences in NF expression and in the stoichiometry of the three NF types depending on the neuronal cell type used and their developmental stage (Perrot et al., 2008; Scott et al., 1985). Moreover, the regulation of the NF subtypes might also differ, with O-GlcNAcylation and phosphorylation having different functions throughout the life of a neuron, so that any imbalance between these two modifications has profound effects on the health of the affected neurons.

Vimentin glycation in ageing

Non-enzymatic glycosylation, known as glycation, is the chemical reaction between an amino acid and a reducing sugar. The vimentin linker regions between the central rod domains are glycation targets in human fibroblasts, and glycation of vimentin filaments leads to the formation of perinuclear aggregates of the glycated vimentin protein, termed aggresomes. These vimentin aggresomes are more abundant in fibroblasts isolated from elderly patients, who have higher levels of vimentin glycation, and they contribute to the impairment of the contractile abilities of fibroblasts, which is a feature of ageing (Kueper et al., 2008; Kueper et al., 2007).

Regulation of IF interactions and assembly by alternative splicing of IF proteins

An additional means of regulating IFs is by alternative splicing. Keratin, vimentin, desmin, lamin, peripherin and GFAP are all alternatively spliced (Landon et al., 1989; Langbein et al., 2010; Lin and Worman, 1993; Park et al., 2000; Zhou et al., 2010). There are multiple splice variants of GFAP, including α, β, δ, ε, γ and κ (Blechingberg et al., 2007; Condorelli et al., 1999; Feinstein et al., 1992; Nielsen et al., 2002; Zelenika et al., 1995) among others. Although GFAP-α is the primary filament-forming variant of GFAP, other GFAP splice variants can be incorporated into the filament network. The ratio between the incorporated GFAP variants is modulated during development and ageing, and aberrations in the GFAP variant composition can have implications for AD and cancer progression (Blechingberg et al., 2007; Middeldorp et al., 2010; Nielsen et al., 2002; van den Berge et al., 2010). Expression of one particular splice variant (GFAP-δ) has been reported to modify GFAP filament assembly, as its presence increases JNK phosphorylation and facilitates increased recruitment of the molecular chaperone αB-crystallin (Perng et al., 2008), which are both indicators of cell stress. Increased expression of GFAP-δ ultimately leads to the collapse of the GFAP network into aggregates that contain αB-crystallin and phosphorylated JNK, suggesting that the relative levels of the GFAP splice variants modulate the cellular stress response.

Peripherin, another neuronal IF found primarily in the peripheral nervous system, also has several splice variants, Per28, Per45 and Per58, with Per45 being required for normal filament formation (McLean et al., 2008). As with GFAP, alterations in the ratio of the different peripherin splice variants in the peripherin network can contribute to disease pathology and can also be used to distinguish between nerve injury in the peripheral and central nervous system (McLean et al., 2010). Per28 is only expressed at low levels under normal conditions, but is upregulated in ALS samples and in neurotoxic aggregates (Xiao et al., 2008). Similar to with GFAP, the aggregation of peripherin splice variants might also be accompanied by an increased recruitment of molecular chaperones [particularly as peripherin can associate with αB-crystallin (Djabali et al., 1997)] and stress-related kinases such as JNK; however, this has yet to be shown in vivo.

Taken together, the above studies indicate that the functions of the IF proteins that have different splice variants might not only be modulated by conventional PTMs. The ratio with which these splice variants are expressed can affect the interactions between filaments and, therefore, the filament surface that is accessible for interactions with other proteins. This can potentially regulate the strength of the interaction between IFs and their target protein, which could then alter the cellular response to different stimuli, such as occurs during stress and disease.

Conclusions

The IF proteins are a diverse family of proteins found throughout the body that, evidently, are regulators of important processes during almost every stage of life. Nestin has emerged as having important functions in myogenesis and cancer. Lamin A might have a role not only in ageing but also in differentiation. Many studies have shown the importance of appropriate IF assembly for cellular function; however, we are now beginning to appreciate that soluble IFs also have their own essential functions in cellular development, as demonstrated for the role of soluble vimentin in neuronal outgrowth. Regulation of IF organization has primarily been thought to be modulated by phosphorylation alone, but the situation is probably more complex, with glycosylation affecting IF organization by regulating IF phosphorylation. Modifying the composition of IFs, for instance by varying the expression of splice variants, can also modify the cellular stress response and, in turn, lead to disease progression. The picture that emerges from the results discussed in this Commentary is that there is a greater complexity in the regulation of IF function and the impact IFs have upon the cell, as indicated by the wide range of diseases that are caused by IF misregulation, than had previously been understood. For the future, unravelling the signalling pathways governing IF expression and regulation, while at the same time examining the impact of IFs on cell signalling, might help us to find appropriately targeted therapies, particularly for neurodegenerative diseases and ageing.

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