Dynamic interplay between the intermediate filament protein nestin and Cyclin-dependent kinase 5

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To my family

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LIST OF ORIGINAL PUBLICATIONS

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I Sahlgren, C.M.*, Pallari, H.-M.*, He, T., Chou, Y.-H., Goldman, R.D. and Eriksson, J.E. 2006. A nestin-scaffold links Cdk5/p35 signaling to oxidant-induced cell death. *EMBO J.* 25:4808-4819.

II de Thonel, A.*, Ferraris, S.E.*, Pallari, H.-M., Imanishi, S.Y., Kochin, V., Hosokawa, T., Hisanga, S., Sahlgren, C. and Eriksson, J.E. 2010. PKCξ regulates Cdk5/p25 signaling during myogenesis. *Mol. Biol. Cell.* 21:1423-1434.

III Pallari, H.-M., Lindqvist, J., Torvaldson, E., Ferraris, S., He, T., Sahlgren, C.M. and Eriksson, J.E. Nestin as a regulator of Cdk5 in differentiating myoblasts. *Manuscript (submitted).*

* Equal contribution.

ABBREVIATIONS

Aβ	β-amyloid peptide
ACh	Acetylcholine
AChR	ACh receptor
AD	Alzheimer's disease
AGJC	Astrocyte gap-junctional communication
AP-1	Activator protein 1
APAF1	Apoptotic protease activating factor 1
Ape1	Apurinic/apyrimidinic endonuclease 1
APP	β-amyloid precursor protein
AR	Amphiregulin
ATM	Ataxia telangiectasia mutated
βAR	β-adrenergic receptor
BFSP	Beaded filament structural protein
BLOC-1	Biogenesis of lysosome related organelles complex 1
BSA	Bovine serum albumine
Cdk	Cyclin-dependent kinase
CHX	Cycloheximide
CKI	Cdk inhibitor
CNS	Central nervous system
CXCR4	CXC chemokine receptor 4
DCX	Doublecortin
DEDD	Death effector domain containing DNA binding protein
DISC	Death inducing signaling complex
dnCdk5	Dominant negative Cdk5
DTT	Dithiothreitol
E	Embryonic day
EBS	Epidermolysis bullosa simplex
ECM	Extracellular matrix
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
ERα	Estrogen receptor α
ERK	Extracellular signal-regulated kinase
ET_BR	Endothelin B receptor
FADD	Fas-associated protein with death domain
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FLIP	FLICE-inhibitory protein
FOXO	Forkhead box protein O
FTCD	Formiminotransferase cyclodeaminase
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GPCR	G protein coupled receptor
GR	Glucocorticoid receptor
GSK-3β	Glycogen synthase kinase 3β
HDAC1	Histone deacetylase 1
IDE	Insulin-degrading enzyme
IF	Intermediate filament
IFAP	IF-associated protein
IKK	IκB kinase
JNK	c-Jun N-terminal kinase
K	Keratin
KO	Knockout
LTP	Long term potentiation
MAPK	Mitogen-activated protein kinase
MDB	Mallory-Denk body
MEF2	Myocyte-specific enhancer factor 2
MF	Microfilament
MHC	Myosin heavy chain
MRF	Myogenic regulatory factor
MT	Microtubule
MTOC	MT organizing center
mTOR	Mammalian target of rapamycin
MuSK	Muscle-specific kinase
NE	Nuclear envelope
NF	Neurofilament
NFκB	Nuclear factor kappa enhancer binding protein
NMDA	N-methyl-D-aspartic acid
NMJ	Neuromuscular junction
NRG	Neuregulin
Oct-1	Octamer transcription factor 1
P	Postnatal day
p130Cas	p130 Crk-associated substrate
p70S6K	p70 ribosomal S6 kinase
PAGE	Polyacrylamide gel electrophoresis
Par	Prostate apoptosis response
PARP	Poly (ADP-ribose) polymerase

PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet-derived growth factor
PDK1	3-phosphoinositide-dependent protein kinase 1
PFA	Paraformaldehyde
PI	Propidium iodode
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C
PKMξ	Protein kinase M ξ
PMSF	Phenylmethylsulfonyl fluoride
PNS	Peripheral nervous system
PP2A	Protein phosphatase 2A
PS	Pseudosubstrate
PSD-95	Postsynaptic density-95
RACK1	Receptor for activated C kinase 1
Rb	Retinoblastoma protein
ROS	Reactive oxygen species
SDF-1	Stromal derived factor 1
SDS	Sodium dodecyl sulphate
SGLT1	Sodium-glucose cotransporter 1
SNAP23	Synaptosome-associated protein
SNARE	SNAP receptor
TBP	TATA binding protein
TGF-β	Transforming growth factor-β
TLC	Thin layer chromatography
TNF	Tumor necrosis factor
TNFR	TNF receptor
TRADD	TNFR1-associated death domain protein
TRAF2	TNFR-associated factor 2
TRAIL	TNF-related apoptosis inducing ligand
TSC2	Tuberous sclerosis protein 2
ULF	Unit-length filament
WT	Wild type
WT1	Wilms' tumor suppressor 1

ABSTRACT

The cellular network of intermediate filaments (IFs) has been long known to be essential for establishing and maintaining cellular organization and tissue integrity. However, the functional versatility of the multimember IF protein family has started to emerge, as a variety of studies have demonstrated the role of IFs in diverse signaling processes. IFs have been shown to interact with receptors, kinases, and other key determinants of signal transduction machinery influencing the operation of signaling determinants in various processes including stress response and tissue growth. The extensive IF network distributed from the nuclear envelope throughout the cytoplasm to the cell membrane provides an enormous surface area to be employed as a protein scaffold. Furthermore, the highly dynamic nature of IFs at the level of both expression and assembly-state renders IFs ideal regulators of cell signaling.

The IF protein nestin is a frequently employed stem and progenitor cell marker, but the physiological functions of nestin have remained largely unknown. An interaction between nestin and the Cyclin-dependent kinase 5 (Cdk5)/p35 signaling complex had been characterized in our laboratory prior to the beginning of this PhD work. Therefore, the aim of this thesis was to investigate the functional significance of nestin in the regulation of the Cdk5/p35 complex. Using oxidative stress-induced neuronal apoptosis as a model system we revealed a prominent antiapoptotic role of nestin deriving from the inhibition of detrimental Cdk5 activity. Through its ability to anchor the Cdk5/p35 signaling complex, nestin regulated the turnover of p35, the calpain-mediated processing of p35 to more stable activator, p25, and the subcellular localization of the Cdk5/p35 complex, thereby having a profound influence on Cdk5 activity and targeting. We succeeded also in illustrating the mechanisms of Cdk5 activation during myoblast differentiation. Atypical protein kinase C ξ (PKCξ) was revealed as a master controller of Cdk5 activity. By means of p35 phosphorylation and calpain activation, PKCξ promoted the calpain-mediated cleavage of p35 to p25, thus enhancing Cdk5 activity. Nestin was observed critical for the onset and pace of myoblast differentiation both of which were significantly accelerated upon nestin downregulation. The absence of nestin induced a substantial increase in the generation of p25 indicating that nestin scaffold sets the pace for myoblast differentiation by affecting the p35 processing and Cdk5 activity. Moreover, we detected a strong bidirectional interrelationship between nestin and the PKCξ-Cdk5 signaling axis in differentiating myoblasts. While nestin appeared to determine the Cdk5 activation, nestin reorganization and stability where dependent on the PKCξ-Cdk5 signaling cascade referring to the ability of Cdk5 to modulate its own activity and targeting by regulating the assembly-state and stability of the nestin scaffold. To summarize, this PhD thesis greatly expands the understanding of the regulatory mechanisms governing the Cdk5 activity introducing nestin and PKCξ as key determinants. Furthermore, this thesis provides novel information about the cellular functions of nestin, of which very little is still known, and reinforces the concept of IFs as signaling scaffolds.

INTRODUCTION

Cell signaling is a communication mechanism enabling the transmission of messages both within the cells and between the cells. It is critical to all aspects of life; development, stress response, regeneration, aging as well as to the maintenance of normal homeostasis. By means of signal transduction cells convert chemical and mechanical stimuli to specific cellular responses. A diverse range of signaling determinants has been characterized and orchestrated into multifactorial signaling pathways converting the initial signals to functional modifications in proteins or changes in gene expression profiles. These modulations have profound influence on the functions of the cell. Disturbances in cell signaling can have detrimental consequences disturbing development, causing diseases, such as cancer, or posing a threat of cell death.

The eukaryotic cytoskeleton, composed of microtubules, microfilaments, and intermediate filaments (IFs), maintains cell shape, enables cell movements and cell division and provides a framework for intracellular trafficking. IFs are encoded by a large number of genes, thereby giving rise to a multimember IF protein family. They have been long known to be essential for establishing and maintaining the mechanical integrity of cells and tissues. However, during the last decade IFs have been proven to interact with a vast number of signaling proteins, thus regulating the functions of these proteins and the operation of the signaling pathways these proteins represent. This intriguing feature of IFs to function as scaffolds for signaling determinants could explain the multiplicity and the complex expression patterns of IF proteins, the latter being determined by tissue type, developmental stage, and environmental signals. IF proteins could provide the cells with customized scaffolding material to adjust the signal transduction specifically for each cell type and for the ongoing physiological process.

The emphasis of this PhD thesis is to illustrate that the functions of IFs extend beyond their role as structural elements and to introduce the elaborate role of IFs as signaling scaffolds. In the experimental part I have investigated the ability of IF protein nestin to function as a regulatory scaffold for the Cyclin-dependent kinase 5 (Cdk5)/p35 signaling complex critical both in development and disease. As my main model systems I have utilized oxidative stress-induced neuronal cell death and myogenic differentiation. In addition, I have examined the dynamic interplay between nestin and Cdk5, paying attention to the ability of Cdk5 to regulate its own nestin scaffold. Furthermore, this thesis describes the upstream regulatory events directing the activation of Cdk5 in differentiating myoblasts. In conclusion, the experimental part of my PhD thesis gives novel insight into the cellular functions of nestin and the regulation of the Cdk5/p35 signaling complex as well as broadens the view on IFs as multifunctional signaling platforms.

REVIEW OF THE LITERATURE

1 Intermediate filaments

Intermediate filaments (IFs) represent one of the three cytoskeletal structures found in nearly all vertebrate cells, the other two being microtubules (MTs) and microfilaments (MFs). IFs are encoded by a large number of genes regulated in an elaborate manner (reviewed by Toivola *et al*., 2005, 2010). Tissue type, developmental stage and various external signals determine the IF content of cells, thus indicating distinct and specialized functions for different IF proteins. Indeed, although IFs are characteristically resistant to mechanical forces and provide cells with mechanical resilience, they are involved in cell signaling (reviewed by Pallari and Eriksson, 2006b). IF proteins specifically interact with critical signaling determinants, thereby perhaps enabling the cell type and developmental stage-specific regulation of signal transduction. The physiological importance of IF proteins is reflected by the fact that mutations in IF genes have been linked to a significant amount of human pathologies (reviewed by Omary, 2009). During the writing process of this thesis, discovered IF mutations were associated with 89 human diseases according to Human Intermediate Filament Database (Szeverenyi *et al*., 2008, http://www.interfil.org). IF-associated diseases affect a broad range of tissues with clinical symptoms varying from skin fragility and liver malfunction to neuronal defects, muscular debility and premature aging (Omary, 2009). These human diseases as well as different mouse models have had a great impact on the knowledge of IF properties and functions.

1.1 The diverse family of intermediate filament proteins

The family of IF proteins is composed of 73 unique gene products. Hence, this large multimember family belongs to the 100 largest gene families in humans (Omary, 2009). The expression of IF proteins is representative for a broad range of metazoans (Kim and Coulombe, 2007). The absence of IF network in plants and fungi is hypothesized to stem from their tough and rigid cell wall providing structural support (Herrmann *et al*., 2009). More simple metazoans often express a limited number of IF proteins, for example nematode *Caenorhabditis elegans* has 12 genes encoding IF proteins in its genome (Carberry *et al*., 2009). Instead, in higher vertebrates the number of IF genes has multiplied during evolution.

IF proteins are grouped into five different sequence homology classes (Table 1). The largest number of IF proteins is categorized as type I and type II keratins expressed in epithelial cells (reviewed by Moll *et al*., 2008). In addition, significant amount of keratins represent hair follicle specific epithelial keratins and hair keratins (Moll *et al*., 2008). At least one type I and one type II keratin is expressed in each cell type as keratins form obligate heteropolymers. Among the most studied keratins are K8 and K18, the major IF proteins in single-layered epithelia found in a variety of organs including liver, kidney, pancreas, lung, and gastrointestinal track. K8/K18 are coexpressed with or without K7/K19/K20 depending on the cell type. Keratinocytes from stratified epithelia express K1/K5/K10/K14, K5/K14 being preferentially expressed in basal keratinocytes and K1/K10 typifying suprabasal keratinocytes in the upper layers of skin. The expression of K6/K16/K17 is triggered in epidermal injuries (Moll *et al*., 2008).

IF protein type	Proteins	Primary cell/tissue type
I	K9-K28	Epithelial cells
	K31-K40	Hair
II	K1-K8	Epithelial cells
	K71-K77	Epithelial cells
	K81-K86	Hair
III	Vimentin	Mesenchymal cells, lens
	GFAP	Glia cells
	Desmin	Muscle
	Peripherin	PNS
	Syncoilin	Muscle, CNS, PNS
IV	NF-L, NF-M, NF-H	CNS, PNS
	α-internexin	CNS
	Nestin	Neuroepithelial cells, muscle
	Synemin	Muscle, CNS
V	Lamins A/C	Nuclear lamina of all cells
	Lamins B1, B2	Nuclear lamina of all cells
VI	BFSP1, BFSP2	Lens

Table 1. The IF protein family.

The expression of type III IF protein vimentin defines the cells of mesenchymal, endothelial and hematopoietic origin (reviewed by Ivaska *et al*., 2007). Muscular cytoskeleton is typified by the type III IF protein desmin (reviewed by Capetanaki *et al*., 2007). Glial fibrillary acidic protein (GFAP) and peripherin, expressed in the astrocytes of central nervous system (CNS) and in the nerve cells of peripheral nervous system (PNS) respectively, are also categorized to type III IF proteins (reviewed by Liem and Messing, 2009). GFAP is known to have splice variants, one of them, GFAP-δ, defining a distinct population of CNS astrocytes giving rise to adult neural stem cells (Roelofs *et al*., 2005). Syncoilin is the most recently identified type III IF protein characterized by three isoforms which are generated through exon splicing (Kemp *et al*., 2009). Syncoilin is expressed in muscle tissue (Kemp *et al*., 2009) as well as in both CNS and PNS (Clarke *et al*., 2010).

The neurofilament (NF) triplet proteins, NF-L, NF-M, and NF-H (for low, medium and high with respect to their molecular masses), the most abundant IF proteins in mature neurons, represent the type IV IF proteins (reviewed by Perrot *et al*., 2008). In addition to NFs, α-internexin, nestin, and synemin are categorized as type IV proteins. α-internexin is expressed in nervous system (Yuan *et al*., 2006) whereas nestin expression defines

neuronal and muscular progenitor cells (Lendahl *et al*., 1990, Sejersen and Lendahl, 1993). Also *synemin* encodes three different protein isoforms through alternative splicing mechanism (Izmiryan *et al*., 2010). Synemin expression was first described in skeletal muscle (Granger and Lazarides, 1980) but has later been reported to occur in the cells of CNS during different stages of development (Tawk *et al*., 2003, Izmiryan *et al*., 2009, 2010).

Type V nuclear lamins are not present in the cytoplasm, but instead are the major components of the nuclear lamina, filamentous layer associated with the inner nuclear envelope (NE; reviewed by Dechat *et al*., 2008). In addition, lamins can be found in throughout the nucleoplasm, although in smaller quantities. A-type lamins (lamins A and C) are encoded by the *LMNA* gene through alternative splicing whereas B-type lamins (lamins B1 and B2) are derived from two different genes, *LMNB1* and *LMNB2* respectively (Dechat *et al*., 2008). Lamins are found in all metazoan cells. Whereas the expression of B-type lamins is induced during the early phases of development, the expression of lamin A/C is executed while differentiation proceeds (Dechat *et al*., 2008). Type VI beaded filament structural proteins 1 and 2 (BFSP1 and BFSP2) constitute the IF network in the eye lens (reviewed by Perng *et al*., 2007, Song *et al*., 2009). BFSP1 and BFSP2 are highly divergent from the other IF proteins as indicated by the sequence alterations and the formation of unique beaded filaments.

1.2 Structure and assembly of intermediate filaments

1.2.1 Structural features of intermediate filament proteins

Albeit drastically differing in primary amino acid sequence, fibrous IF proteins share a common tripartite domain organization (Figure 1; reviewed by Kreplak *et al*., 2004, Parry *et al*., 2007, Herrmann *et al*., 2007, 2009). They all share the central, α-helical rod domain flanked by the non-α-helical amino (N) and carboxy (C)-terminal regions. The highly conserved rod domain, common denominator of the IF protein family, display pronounced repeats of heptads, where the first and the fourth positions are occupied by an amino acid with hydrophobic side chain. These repeats are responsible for the generation of a coiled-coil dimer, the initial assembly step of IFs formed by two IF proteins wound around each other. The rod domain is divided into four subhelices termed coil 1A, coil 1B, coil 2A, and coil 2B (Kreplak *et al*., 2004, Parry *et al*., 2007, Herrmann *et al*., 2007, 2009). These are interrupted by linker domains L1, L12, and L2, the structures of which remain elusive. Linkers have been suggested to serve as hinges promoting the flexibility of a coiled-coil dimer. Stutter is a conserved sequence of four amino acids inserted in the coil 2B. Highly conserved sequences are pinpointed at both ends of the rod domain. Mutations in these highly conserved sequences inhibit the generation of functional IF network (Kreplak *et al*., 2004, Parry *et al*., 2007, Herrmann *et al*., 2007, 2009).

Contrary to the conserved rod domain consisting of ~310 amino acids, the distal regions show considerable variation in both size and amino acid composition, thus contributing to the diversity of the IF protein family. The lengths of the N-terminal head domains vary from few amino acids in human nestin to 167 amino acids observed in K5. The variation in the C-terminal tail domains is even more pronounced as indicated by K19 with a 15 amino acid long tail and human nestin with a tail domain longer than 1300 amino acids

(Herrmann *et al*., 2009). These less conserved domains have been suggested to be involved in the specific functions of individual IF proteins. The nuclear lamins are known to differ structurally from cytoplasmic IF proteins (Dechat *et al*., 2008). Within their C-terminal tail they have a structural motif resembling immunoglobulin fold and a nuclear localization signal responsible for nuclear transport. Furthermore, lamins are expressed as pre-lamins which are subjected to modification and processing during protein maturation (Dechat *et al*., 2008).

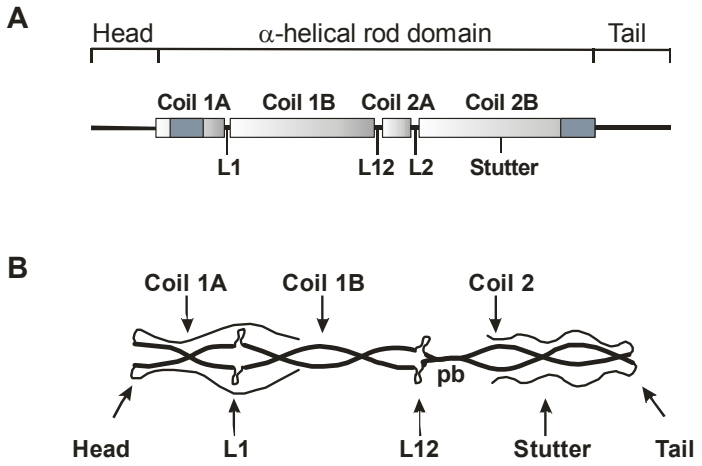

Figure 1. Domain organization of an IF molecule (A) and IF dimer (B). (A) The figure represents the prevailing model of an IF molecule. Boxes represent the four subhelices of the central rod domain, coil 1A, coil 1B, coil 2A, and coil 2B, whereas the grey segments denote the highly conserved sequences. Subhelices are separated by the linker regions L1, L12, and L2. Stutter is the conserved segment inserted in the coil 2B. "Head" and "tail" indicate the non-α-helical distal regions. (B) The model depicts the structure of a vimentin-like IF dimer. According to the most recent crystallographic studies, the segments termed coil 2A, L2, and coil 2B appear to constitute a continuous coiled-coil, coil 2, with a short paired bundle (pb) formed by the segments coil 2A and L2. Positively charged distal domains bend towards the more negatively charged rod domain (modified from Herrmann *et al*., 2009).

1.2.2 Assembly of intermediate filaments

The assembly of IFs is initiated when the rod domains of two IF proteins interact in a parallel fashion to form a coiled-coil dimer (Figure 1; reviewed by Kreplak *et al*., 2004, Parry *et al*., 2007, Herrmann *et al*., 2007, 2009). Dimers interact to form anti-parallel half-staggered tetramers that interact laterally to form unit-length filaments (ULFs; Figure 2). One ULF is composed of an average of eight tetramers. After the longitudinal end-to-end annealing of ULFs to longer filaments, occurs a phase of radial compaction when the filament diameter is reduced from approximately 17 nm to 10 nm due to molecular rearrangements. The 10 nm diameter reflects mature IFs and clarifies the origin of the name "intermediate filament" falling in between 25 nm diameter MTs and 9 nm diameter MFs. The assembly of nuclear lamins differs from the cytoplasmic IFs involving simultaneous lateral and longitudinal interactions between dimers with tendency to head-to-tail interactions (Kreplak *et al*., 2004, Parry *et al*., 2007, Herrmann *et al*., 2007, 2009). Besides the numerous *in vitro* studies, carried out to thoroughly understand the distinct steps of IF assembly, IF formation has been intensively surveyed *in vivo.* Different

structural forms of IFs have been identified in cell model systems. Type III IF proteins have been shown to exist as non-filamentous particles, short filaments and long filaments (reviewed by Kreplak *et al*., 2004, Goldman *et al.*, 2008). During the initial phases of cell spreading on substratum the IF particles are connected to short filaments that subsequently become linked to longer IFs. Although the exact nature of IF particles and short filaments is unknown it has been proposed that the non-filamentous particles would be composed of ULF-like precursors which, in turn, would anneal to produce longer filaments (Kreplak *et al*., 2004, Goldman *et al.*, 2008).

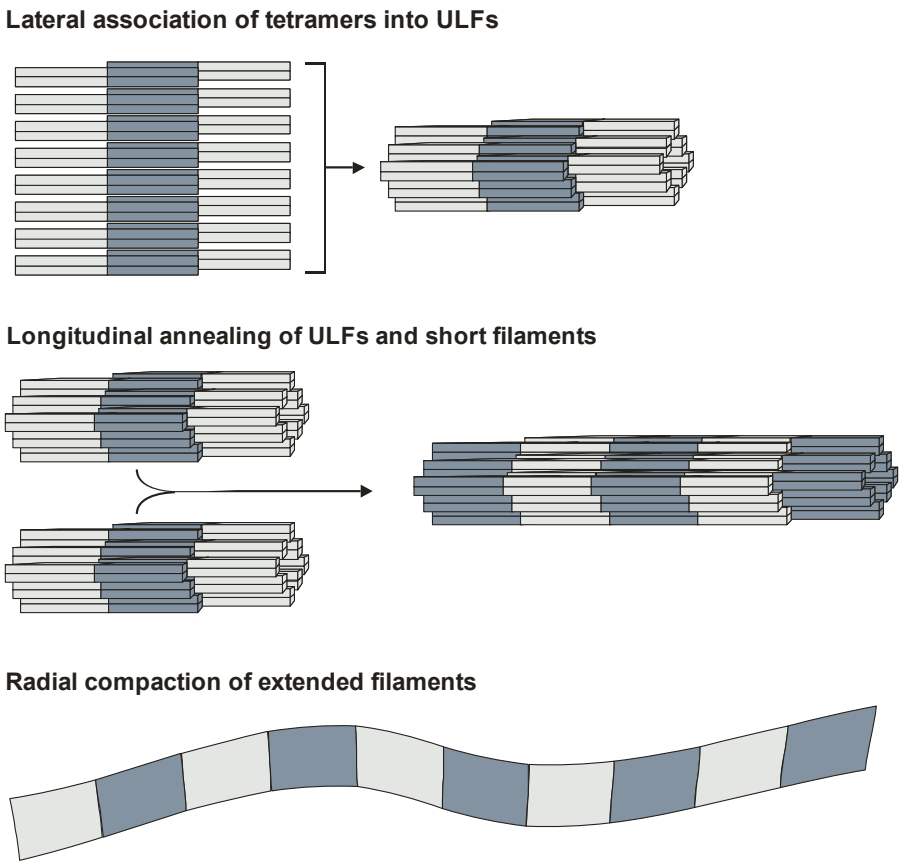

Figure 2. Schematic presentation of the phases of cytoplasmic IF assembly. The assembly of cytoplasmic IFs can be divided into three major phases. In the first phase an average of eight tetramers associates laterally into ULFs, followed by a second phase where ULFs and short filaments anneal longitudinally to longer filaments. The mature IF structure is accomplished in the third phase with the radial compaction of filaments (adapted from Herrmann *et al*., 2009).

In contrast to most type III IF proteins assembling into homopolymers, keratins form obligate heteropolymers containing one acidic (type I) and one basic (type II) keratin. The loss of one keratin leads to the instability of the partner (Strnad *et al*., 2008). Type IV proteins nestin and synemin form filaments with type III IF proteins (Bellin *et al*., 1999, Steinert *et al*., 1999, Jing *et al*., 2007). Considering the established importance of the N-terminus in IF assembly (Herrmann *et al*., 1996, Mücke *et al*., 2004) and the short head

domains of nestin (Steinert *et al*., 1999) and synemin (Khanamiryan *et al*., 2008) it seems evident that nestin and synemin do not assemble into homopolymers. NF triplet proteins exhibit a complex assembly pattern where four IF proteins are incorporated into the same filament, the fourth being α-internexin or peripherin (Perrot *et al*., 2008). The dissimilarities in assembly processes are expected to prevent the formation of mixed IFs within the cells (Herrmann *et al*., 2009).

In addition to the amount of subunits, there are fundamental differences in the assembly and properties of the three cytoskeletal networks. MTs and MFs are polarized structures enabling vesicular transport along them whereas cytoplasmic IFs are non-polar due to the antiparallel interaction between dimers (Herrmann *et al*., 2007). The polymerization of MT and MF subunits, tubulin and actin respectively, occurs on both ends of the filaments, one end growing more rapidly than the other. IFs lengthen by end-to-end annealing of ULFs or longer filaments but exhibit also intercalary subunit exchange along the length of entire filament indicating a topologically open architecture (Colakoğlu and Brown, 2009). Furthermore, the ability of IFs to polymerize into filaments requires neither energy nor accessory cofactors contrary to MTs and MFs the dynamics of which are depended on nucleotide hydrolysis. Different biophysical analyses have given insight into the special features of IFs (reviewed by Herrmann *et al*., 2007, Kreplak and Fudge, 2007). It has been long known that IFs remain highly insoluble in buffers with high salt concentration or ionic detergents where MTs and MFs become disassembled. Moreover, accumulating evidence implies that the ability of IFs to resist applied forces is increased compared to MTs and MFs. A phenomenon of strain stiffening has been described with IFs. When mechanical stress is directed to IFs, filaments are not broken but instead become viscoelastic showing extensibility that can not be seen with MTs and MFs (Herrmann *et al*., 2007, Kreplak and Fudge, 2007). The mechanical properties of filaments composed of distinct IF proteins differ, thus indicating that the selection of IF proteins may reflect the mechanical stress experienced by the tissue (Schopferer *et al*., 2009).

1.3 Intermediate filaments maintain tissue integrity and cytoplasmic organization

The IF network is not a separate structural entity but instead IFs appear to form a cellular framework closely connected to other cytoskeletal systems as well as to different structural elements and cell organelles. IF-associated proteins (IFAPs) are utilized as linker proteins and mediate many of these interactions (reviewed by Green *et al*., 2005). Periplakin, plectin and desmoplakin, proteins of the plakin family, are well-established in bridging IFs to MTs, MFs, adhesion sites, and cell organelles (Figure 3; reviewed by Sonnenberg and Liem, 2007). The essential functions of IFs, abilities to maintain the integrity of the cells and tissues and to provide organization for cell interior, are dependent on IF interactions.

IFs interact closely with different cell membrane structures. Keratins are tethered to desmosomes, complexes at cell-cell junctions, and to hemidesmosomes, cell-substratum contacts. Desmosomes utilize Ca^{2+}-dependent adhesion molecules, desmogleins and desmocollins for cell-cell interaction. The tails of these proteins associate with plakoglobin and plakophilin, both of which, in turn, interact with the plakin family members linking keratin cytoskeleton to the junctions. At hemidesmosomes

plectin connects keratin filaments to integrins (reviewed by Sonnenberg and Liem, 2007, Green and Simpson, 2007). Keratin interactions to cell membrane structures are important in the formation of a well-anchored network stabilizing epithelial tissues. This became evident when the symptoms of the skin blistering disease epidermolysis bullosa simplex (EBS) were realized to derive form mutations in epidermal K5 and K14 (Coulombe *et al*., 1991, reviewed by Pekny and Lane, 2007, Coulombe *et al*., 2009). As a consequence of these mutations, the most severe ones enhancing keratin aggregation, skin keratinocytes are extremely fragile and the patients suffer skin blistering. Stretch induced aggregation of K14 bearing EBS-mutation was connected to a subsequent collapse of cell-cell and cell-substratum contacts (Russell *et al*., 2004). Hence, the interactions with adhesion structures seem to define the capacity of keratins to resist and distribute the forces of mechanical stress throughout the epithelial tissue (Green and Simpson, 2007). Considering the general properties of IFs, being extended and strong although flexible, IFs can be stated ideal for providing mechanical resilience to cells and tissues (Goldman *et al*., 2008).

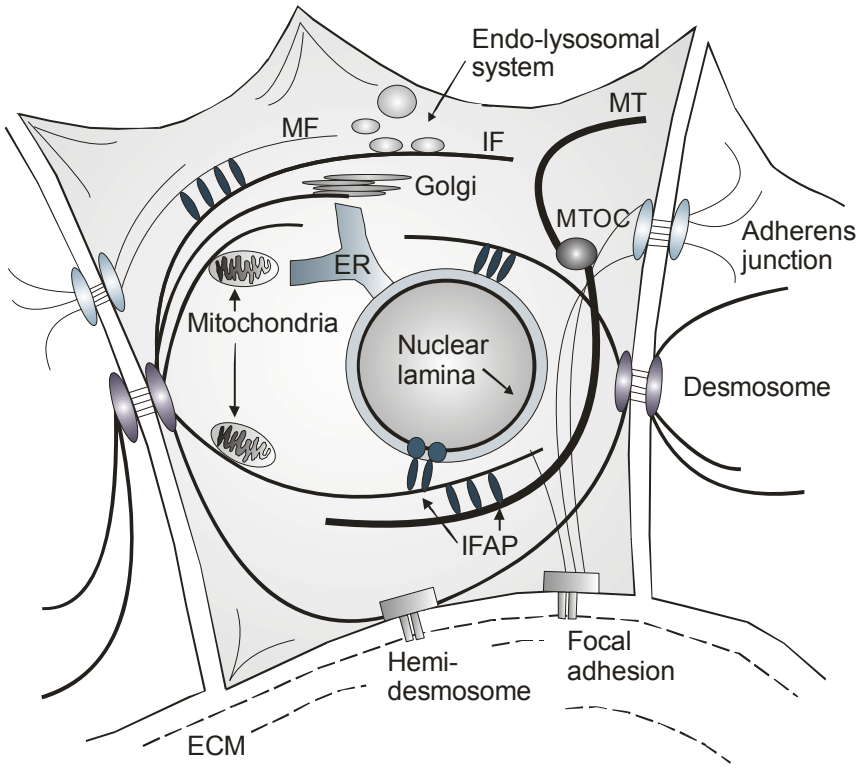

Figure 3. Hypothetical cell depicting the role of IFs in tissue stability and cytoplasmic organization. The three cytoskeletal systems, MTs, MFs and IFs, are connected to each other by IFAPs. IFs are anchored to desmosomes at cell–cell junctions and hemidesmosomes at cell–matrix junctions. The transmembrane proteins mediating the association between the neighboring cells and with the extracellular matrix (ECM) are cadherins and integrins, respectively. In addition, IFs are coupled to the NE and lamins through IFAPs and lamin/NE-binding proteins. IFs anchor cell organelles, mitochondria and the Golgi apparatus, as well as work as a regulatory scaffold for vesicular transport systems. (ER, endoplasmic reticulum; MTOC, microtubule-organizing center; modified from Herrmann *et al*., 2007.)

In cardiac muscle desmin is tethered to intercalated discs, adhesion structures connecting the adjacent cells in myocardium (reviewed by Capetanaki *et al*., 2007). Desmin filaments seem to regulate the cellular organization rather comprehensively by integrating key elements of muscle tissue. Distinct plectin isoforms anchor desmin to costameres, protein complexes on muscle plasma membrane, and to myofibrils, thereby connecting the cell membrane and the contractile apparatus (Konieczny *et al*., 2008). Synemin is similarly tethered to adhesive structures, but instead of binding to IFAPs, the long C-terminal tail of synemin interacts directly with vinculin and talin localized on costameres as well as with α-actinin at Z-discs (Bellin *et al*., 1999, Sun N *et al*., 2008a, 2008b). These interactions of synemin have been suggested to strengthen the association of desmin/synemin heteropolymers to costameres. As desmin integrates the functional elements of muscle tissue and is suggested to participate in the transmission of contractile forces, it is no surprise that desmin mutations lead to severe malfunctions, cardiac and skeletal myopathies (reviewed by Paulin and Li, 2004, Goldfarb and Dalakas, 2009).

IFs extend from the NE throughout the cytoplasm to the cell membrane. The outer nuclear membrane protein nesprin-3 associates with plectin, thereby enabling the anchorage of IFs to the NE (Wilhelmsen *et al*., 2005). As plectin mediates the IF interactions to cell-cell and cell-matrix junctions (Bhattacharya *et al*., 2009) this finding proposes that by utilizing IFAPs, IFs connect the nucleus and cell adhesion sites. The ablation of A-type lamins, important regulators of NE organization, led to a loss of nesprin-3 from the NE and disturbances in vimentin filaments (Houben *et al*., 2009), hence demonstrating the functional link between the cytoplasmic IFs and the nuclear lamina. The anchorage of nucleus to the cytoskeleton and cell membrane has been stated to protect nuclei from external mechanical forces, mediate mechanotransduction, i.e. a process where mechanical stimuli evokes cellular signals, and modulate the reorientation of nucleus during cell migration (Dechat *et al*., 2008, Houben *et al*., 2009).

An important function of IFs is to maintain the internal organization of cells through interactions with cell organelles. Furthermore, accumulating data points to the role of IFs as important regulators of organelle topography, shape and function. Mitochondria have been functionally linked to the IF network. The ablation of desmin causes a hyperproliferation of mitochondria together with abnormal positioning, morphological alterations and reduced respiration rate, the effects being most prominent in cardiac muscle (Milner *et al*., 2000). Likewise vimentin filaments are depicted to interact with and regulate the morphology and cellular distribution of mitochondria (Tang *et al*., 2008). Also K8 knockout (KO) hepatocytes are characterized with abnormalities in mitochondrial morphology, distribution and function (Tao *et al*., 2009). Thus far it is not clear how IFs interact with mitochondria, although plectin has been proposed to mediate the interaction (Konieczny *et al*., 2008). In addition, the reasons for altered mitochondrial functions remain obscure. IFs have been speculated to provide structural support for the mitochondrial membrane or balance mitochondrial fission and fusion or, alternatively, regulate the functionality of mitochondria through protein targeting to the mitochondrial membrane (Capetanaki *et al*., 2007). Among IF interacting cell organelles is also the Golgi apparatus which has been shown to associate with vimentin IFs through an interaction between vimentin and Golgi-associated enzyme formiminotransferase cyclodeaminase (FTCD; Gao and Sztul, 2001). Ectopically expressed FTCD colocalizes with vimentin IFs causing Golgi fragmentation and association of Golgi elements along vimentin. Moreover, in vimentin downregulated cells the polarization of Golgi complex towards the direction of migration was reported to be disturbed, further indicating the

ability of vimentin to affect the Golgi apparatus (Phua *et al*., 2009). In addition, the loss of an intact keratin network in epithelial cell lines due to the expression of K18 where arginine 89 has been mutated to cysteine (R89C) incurred the redistribution of Golgi (Kumemura *et al*., 2004). However the relevance of keratin-Golgi association has been questioned as no apparent Golgi abnormalities are present in hepatocytes and colonocytes in K8 KO or K18 R89C transgenic mice (Toivola *et al*., 2005).

Cytoplasmic IFs have also been implicated in vesicular transport. Besides the interplay between IFs and the endo-lysosomal sorting machinery (discussed in section 2.3) IFs have been depicted to regulate the distribution of melanosomes. K5 plays a role in the transfer of melanosomes from melanocytes to the upper layers of epidermis as exemplified by a hyperpigmentation disorder, Dowling-Degos disease, arising from a K5 mutation (Planko *et al*., 2007). In addition, the dynamic changes observed in vimentin networks in *Xenopus laevis* melanophores in response to hormone stimulation have been associated with the distribution of melanosomes to the cell periphery (Chang *et al*., 2009). It was suggested that vimentin, forming a cage-like network around melanosomes would coordinate the melanosome movements and that modulation of vimentin filaments would be prerequisite for the subcellular transfer of melanosomes. To summarize, IFs are connected to a variety of cellular elements, hence being essential in integrating tissues, providing mechanical resilience and controlling intracellular organization.

1.4 Dynamic nature of intermediate filaments

Although IFs are essential in stabilizing tissues and providing subcellular organization, they are far from static structures. The dynamic nature of IFs can be observed on multiple levels ranging from the variable expression patterns of IF proteins to the rapid structural modifications on mature IFs.

1.4.1 Physiological and pathological expression patterns of intermediate filament proteins

The expression patterns of IF proteins are strictly regulated. Distinct IF networks typify particular cell and tissue types (Table 1), but the transcription of IF genes can vary according to the developmental stage or in response to stress or injury. For example, a transition in cellular IF content occurs during the maturation of neurons when vimentin and nestin present in neuronal progenitor cells are gradually replaced by NFs (Yabe *et al*., 2003). Similarly desmin displaces nestin and vimentin during muscle development (Sejersen and Lendahl, 1993). In epidermis the keratinocytes switch their IF gene expression while moving from the proliferative basal cell layer to the postmitotic suprabasal cell layer during epidermal differentiation (Moll *et al*., 2008). K5/K14 in basal keratinocytes are changed to K1/K10 in suprabasal cells in the upper layers of skin. These changes in the IF expression most probably reflect the altered environmental conditions and cellular requirements to which cells can answer by modulating the IF network. Considering the widespread functions of IFs in providing mechanical resilience (section 1.3) and mediating the signal transduction (section 2), modifications in the IF network appear a practical mechanism to adapt to novel conditions.

Tissue regeneration is depicted in several context to correlate with alterations in the expression of IF proteins. For instance, the expression of K6 and K16 is rapidly induced

in keratinocytes at the skin wound edge. The importance of K6/K16 is suggested to stem from their ability to provide the keratinocytes for adequate plasticity required for migration to wound area and mechanical strength to survive in wounded tissue (Wong and Coulombe, 2003). In certain situations, the IF genes upregulated following an injury are the ones transcribed during the early stages of development. Vimentin and nestin are replaced by GFAP in maturating astrocytes, but their expression can be switched on upon CNS injuries, when astrocytes form a glial scar at the site of injury (Pekny *et al*., 1999). Similarly, enhancement in the expression of vimentin and nestin occurs in kidney podocytes in response to nephrosis (Zou *et al*., 2006) and in skeletal muscle following an injury (Vaittinen *et al*., 2001). This could reflect the enhanced capability of progenitor-type cells to survive in injured tissue and mediate regeneration. Furthermore, several forms of stress ranging from mechanical stress and heat to viral infections and toxins are known to upregulate either resident or *de novo* IF proteins in multiple tissues (reviewed by Toivola *et al*., 2010). The ablation of IF proteins depicted in regenerative processes including vimentin, GFAP and K6/K16, has been demonstrated to compromise the response to stress or injury (Eckes *et al*., 2000, Wong and Coulombe, 2003, Wilhelmsson *et al*., 2004). Therefore, the transition of cellular IF content can be a clear advantage for cells to survive in challenging environment.

1.4.2 Assembly dynamics of intermediate filaments

In addition to the variable expression patterns of IF proteins, the assembly of IFs *in vivo* is a highly dynamic process, non-filamentous IF particles/precursors displaying seemingly motile behavior. Upon cell plating, vimentin precursors become rapidly transported to the peripheral regions of spreading cells where they are gradually converted first to shorter filaments (squiggles) and then to longer filaments (Prahlad *et al*., 1998). The distribution of vimentin precursors and squiggles within the cells is dependent on the MT network and involves an interaction between vimentin and kinesin, motor protein responsible for the anterograde transport (Prahlad *et al*., 1998). The proper assembly and maintenance of the type III IF networks requires bidirectional MT-dependent transport, hence relying also on the dynein/dynactin-mediated retrograde movements (Helfand *et al*., 2002). During neurite outgrowth dynein-binding protein Ndel1 facilitates the interaction of vimentin with dynein-complexes (Shim *et al*., 2008). This interaction seems particularly important during axon regeneration when cytoskeleton is being remodeled (Toth *et al*., 2008). Similarly to the type III IFs, the motility of type IV IFs in neurons is regulated by the MT system and molecular motors (Yabe *et al*., 1999, 2000, Prahlad *et al*., 2000, Shah *et al*., 2000, Helfand *et al*., 2003a).

As the three cytoskeletal systems closely cooperate, it is not surprising that disturbing MFs impedes the motility of vimentin precursors (Yoon *et al*., 1998). Interestingly, it seems that the intracellular movements of different IF precursors are differentially regulated. The mobility of keratin squiggles is slower, predominantly directed to cell interior and less dependent on MTs than what detected with vimentin precursors (Yoon *et al*., 2001, Wöll *et al*., 2005). Instead, keratin precursors are abundant at focal adhesion in lamellipodia from where they migrate inwards along MFs subsequently elongating to longer assembly intermediates to become integrated into the peripheral keratin network (Windoffer *et al*., 2006, Kölsch *et al*., 2009). These observations indicate that IF polymerization can be spatially regulated by means of IF precursor transport and the formation of IF assembly intermediates can be restricted to specific cellular compartments, such as adhesive structures, hence probably enhancing cytoskeletal adjustment during cell migration (Windoffer et al., 2004, 2006, Kölsch *et al*., 2009). An

intriguing way of cells to regulate the local IF content is the dynamic cotranslation described for peripherin. Peripherin mRNA-containing particles move along MT tracks while protein synthesis can be initiated when the particles stop and peripherin becomes cotranslationally assembled into IF precursors (Chang *et al*., 2006). These translational units are suggested to concentrate the IF protein synthesis and IF polymerization to specific cellular compartments.

1.4.3 Turnover and remodeling of intermediate filaments

After being constructed, IF networks are exposed to constant turnover and remodeling. In late 1980s it started to become evident that a small portion of IF proteins/precursors remains in unpolymerized soluble pool within the cells (reviewed by Eriksson *et al*., 1992, 2009). IFs, previously considered extremely static structures, were discovered to undergo dynamic subunit exchange i.e. a process where polymerized IF subunits are being replaced by soluble IF proteins/precursors which become incorporated into the existing IF network. Moreover, IFs were reported to be subjected to remarkable reorganization during differentiation, stress and pathological situations (for example Tokuyasu *et al*., 1984, Perry *et al*., 1985, Liao *et al*., 1995, reviewed by Toivola *et al*., 2010). Important insight into the dynamic nature of IFs was obtained from the studies showing the mitotic disassembly of IF network. IFs undergo marked structural changes during cell division ranging from the almost total disassembly of nuclear lamina (reviewed by Foisner, 1997) to less dramatic reorganization of cytoplasmic IFs around the mitotic apparatus (reviewed by Chou *et al*., 2007). Furthermore, various motile processes, many of them associated with alterations in cell morphology, are characterized by IF remodeling. For example in lymphocytes, profound changes in vimentin filaments have been presented to occur upon lymphocyte transmigration across the vascular endothelium (Nieminen *et al*., 2006). The continuous subunit exchange and the structural modulation of the IF network require an efficient regulatory mechanism that can be specifically activated depending on the ongoing physiological process.

1.4.4 Phosphorylation as a regulator of intermediate filament dynamics

A distinguishing feature of IFs is their tight regulation by posttranslational modifications, phosphorylation being the most prominent. The initial observations of IF protein phosphorylation were made more than 30 years ago when NFs (Pant *et al*., 1978) and keratins (Sun and Green, 1978) were described to undergo this modification. Nearly a decade later phosphorylation of vimentin was reported to cause the disassembly of vimentin filaments *in vitro* (Inagaki et al., 1987). Thereafter IF phosphorylation has been extensively studied and phosphorylation has been demonstrated to be the main regulator of IF assembly, organization and turnover (reviewed by Omary *et al*., 2006, Pallari and Eriksson, 2006a). IF phosphorylation is a key feature of multiple cellular activities, such as mitosis, tissue development and cell motility, all of which are characterized by significant modifications in IF structure (Table 2). The phosphorylation-mediated reorganization of IF network enables the cell division (Foisner, 1997, Sihag *et al*., 2007). Nestin phosphorylation is a prerequisite for the reorganization of nestin filaments upon myoblast fusion (Sahlgren *et al*., 2003) whereas the phosphorylation of NFs regulates their transport and organization in maturating axons (reviewed by Sihag *et al*., 2007, Perrot *et al*., 2008). The phosphorylation of vimentin controls the vesicular trafficking of integrins to the cell membrane, thereby regulating the directional motility of the cell (Ivaska *et al*., 2005).

Phosphorylation seems to be exclusively targeted to the head and tail domains of IF proteins, as exemplified by numerous *in vitro* and *in vivo* studies (Omary *et al*., 2006, Pallari and Eriksson, 2006a). The most likely reason for this specificity is the limited accessibility of kinases to the central rod domain (Omary *et al*., 2006). Moreover, the N- and C-terminal regions showing variability in both length and amino acid sequence are assumed to be responsible for the specific functions of IF proteins. N-terminus is also recognized as an important mediator of IF assembly and filament strength (Herrmann *et al*., 1996, Mücke *et al*., 2004). For example, the N-terminal head domain of vimentin contains a number of positively charged arginines. Hence, the N-terminus has been suggested to bend towards the highly negatively charged rod domain, thereby enabling the lateral aggregation of tetramers (Kreplak *et al*., 2004). It could be estimated that a switch in the N-terminal charge due to phosphorylation might hamper the ULF formation, thus inflicting filament disassembly. Indeed, the remarkable preference of N-terminal phosphorylation in IF disassembly has become evident from numerous pieces of work (reviewed by Pallari and Eriksson, 2006a). C-terminal phosphorylation of IFs is less general and has been implicated, in terms of structural influences, mainly with IF proteins having divergent distal regions, such as nestin (Sahlgren *et al*., 2001, 2003), or different assembly behavior, such as lamins (Heald and McKeon, 1990). Today it is known that the phosphorylation patterns of IF proteins are relatively complex, individual phosphorylation sites having context-dependent roles.

Mitosis is not the only physiological process during which IF phosphorylation and subsequent reorganization can be detected (Table 2). Different forms of stress and exposure to toxic compounds enhance keratin phosphorylation (Liao *et al*., 1997, Toivola *et al*., 2002) although the purpose of phosphorylation is not necessarily related to structural modifications on filaments. Instead, keratin IFs have been hypothesized to work as phosphate sponges protecting cells from excess of stress-associated kinase activity (Omary *et al*., 2006). Mechanical shear stress of lung alveolar epithelial cells is reported to cause phosphorylation-dependent disassembly of keratin IFs (Ridge *et al*., 2005). However, when only a moderate shear stress was applied to cells for a short period of time, phosphorylation was noticed to be associated with bundling of keratin filaments into thicker tonofibrils, hence indicating that phosphorylation can also reinforce IFs and possibly enhance the ability of cells to resist mechanical stress (Flitney *et al*., 2009). Furthermore, phosphorylation affects IF dynamics by regulating IF protein turnover and selective degradation. GFAP is discovered to undergo more rapid turnover when the N-terminal phosphorylation sites are mutated to alanine (Takemura *et al*., 2002). Similarly, hyperphosphorylation is reported to protect K18 from caspase-dependent degradation (Ku and Omary, 2001). K18 and the binding partner K8 are targeted to ubiquitylation and proteosomal degradation with the help of which, for example, the imbalance in K8/K18 protein levels can be corrected (Ku and Omary, 2000). Phosphorylation of K8 on serine 74 (S74; according to the new nomenclature, Omary *et al*., 2009) was observed to have a stabilizing effect yet following shear stress the same phosphorylation site triggered the ubiquitylation and proteosomal degradation of K8 in alveolar epithelial cells (Jaitovich *et al*., 2008). Differences in susceptibility for ubiquitylation might stem from different cell types or biological processes determining the enzymes in the ubiquitin-proteasome pathway.

When referring to IF phosphorylation, one has to remark that phosphorylation can also have outcomes not related to structural remodeling or protein turnover. Importantly, IF phosphorylation is known to regulate the interactions between IFs and different signaling determinants, thus having an influence on signal transduction (Table 2, reviewed by

Hyder *et al*., 2008). Moreover, several IF kinases have been demonstrated to interact with IF substrates. These interactions have often functional consequences that can be observed as a sequestration-based regulation of the kinases (Omary *et al*., 2006, Pallari and Eriksson, 2006b).

IF property or function affected by phosphorylation	Examples
IF assembly-state and organization	Solubilization and structural remodeling of IF network during mitosis, stress, differentiation etc (Foisner, 1997, Sahlgren *et al*., 2003, Ridge *et al*., 2005, Omary *et al*., 2006, Sihag *et al*., 2007)
	Keratin bundling during moderate shear stress (Flitney *et al*., 2009)
IF transport and cellular localization	Transport of NFs from the cell soma to axons (Sihag *et al*., 2007, Perrot *et al*., 2008)
IF turnover	Protection of IF proteins form proteosomal and caspase-dependent degradation (Ku *et al*., 2000, Ku and Omary, 2001, Takemura *et al*., 2002)
	Targeting of keratins to proteasomes (Jaitovich *et al*., 2008)
IF-mediated protein sequestration and targeting	IF-mediated sequestration of 14-3-3 proteins (Ku *et al*., 1998a, Tzivion *et al*., 2000, Kim *et al*., 2006)
	Association of IFs with kinases and other signaling molecules (Sin *et al*., 1998, Ku *et al*., 2004, Chou *et al*., 2009)
	Vimentin-dependent trafficking of integrins to the cell membrane (Ivaska *et al*., 2005)
Ability of IFs to protect cells from apoptosis	Keratin-dependent protection of cells from stress-induced kinase activity (Ku *et al*., 2007)

Table 2. IF phosphorylation regulates a variety of IF properties and functions. Examples related to the IF signaling functions are discussed in section 2 (modified from Omary *et al*., 2006).

2 Intermediate filaments as versatile signaling platforms

Until the last decade, IFs were considered mainly as structural proteins. However, the functional versatility of IF protein family has been revealed by multiple studies pointing to the role of IFs in sequestration-based regulation of signaling determinants. The extensive IF network distributed from the NE throughout the cytoplasm to the cell membrane provides an enormous surface area to be utilized as a scaffold for various proteins (Goldman *et al*., 2008). Furthermore, the highly dynamic nature of IFs, at the level of both expression and assembly, makes IFs ideal signaling factors. Disassembly of filaments can have tremendous effect on the signaling determinants regulated by the

IF scaffold. In response to developmental, stress-related or regenerative signals IF network can be replaced or supplemented with other IF proteins, more suitable to the prevailing needs in terms of signal transduction. Hence, the purpose of the multimember IF protein family could be explained by the demand of specialized regulatory elements for distinct physiological processes.

2.1 Intermediate filaments regulate cell death and survival pathways

2.1.1 Intermediate filaments control multiple components in apoptotic machinery

The ability of IFs to help cells to tolerate stress extends beyond their role as structural support against mechanical forces, since emerging evidence imply that IFs provide resistance against apoptotic stimuli. Diverse molecular mechanisms of IF-dependent cytoprotection have been described, many of them relating to the regulation of cell death and survival pathways. An entity of IF-regulated signaling determinants is composed of proteins associated with apoptotic death receptor pathways (Figure 4). The IF-mediated regulation governs proteins from the level of receptors to the downstream executioners of apoptosis, caspases.

Alterations in keratin IFs in liver have been demonstrated to render mice more prone to Fas-mediated liver damage (Ku *et al*., 2003). Fas receptor was linked to the IF network when a failure in Fas receptor targeting to the cell membrane was observed in K8 KO hepatocytes (Gilbert *et al*., 2001). The ablation of K8/K18 IFs leads to an increased density of Fas receptor at the cell membrane suggesting a role for K8/K18 in modulating the Fas receptor distribution. K8/K18 filaments have been reported to bind also the cytoplasmic domain of tumor necrosis factor (TNF) receptor 2 (TNFR2; Caulin *et al.*, 2000). The lack of K8/K18 in epithelial cells increases their sensitivity towards TNF-induced cell death (Caulin *et al.*, 2000). K8/K18-deficient cells show higher activities of c-Jun N-terminal kinase (JNK) and nuclear factor kappa enhancer binding protein (NFκB) when treated with TNF indicating that K8/K18 IFs modulate the TNF-induced signaling (Caulin *et al.*, 2000) although the detailed mechanism remains still uncharacterized.

IFs appear critical also for the formation of death inducing signaling complex (DISC) at death receptors. K18 has been proposed to regulate the recruitment of TNFR1-associated death domain protein (TRADD) to activated TNFR1 as overexpressed K18 is able to attenuate TNF-induced apoptosis through the sequestration of TRADD (Inada *et al*., 2001). K17 modulates TNF-signaling through similar TRADD scaffolding in skin keratinocytes (Tong and Coulombe, 2006). Interestingly, the transition to the stage of catagen, apoptosis-driven involution in the hair growth cycle, when distinct hair follicle cells are eliminated in a TNF-dependent manner, is premature in K17 KO mice resulting hair cycling defect and alopecia (Tong and Coulombe, 2006). DISC formation is more prominent also in K8 KO hepatocytes as indicated by the increased recruitment of Fas-associated protein with death domain (FADD) and procaspase-8 to Fas receptor upon stimulation (Gilbert *et al*., 2008). In addition, K8/K18 filaments have been established to bind to death receptor-associated caspase-8 inhibitor, cellular FLICE-inhibitory protein (c-FLIP; Gilbert *et al*., 2004). c-FLIP has a major influence on apoptotic response as it, by being recruited to DISC, can restrain the caspase-8 activation. Moreover, c-FLIP is known to act as a platform for Raf-1, an upstream regulator of the extracellular signal-regulated kinase (ERK) pathway (Kataoka *et al*., 2000). Consequently, the drastic reduction of c-FLIP in K8 KO cells (Gilbert *et al*., 2004) could partially explain the

increased caspase activity and weaker Fas-induced antiapoptotic ERK signaling in K8 KO hepatocytes (Gilbert *et al*., 2001, 2004, 2008).

As dramatic cytoskeletal disassembly is associated with apoptotic cell death, the proteolytic processing of IF proteins including K18 (Caulin *et al*., 1997), vimentin (Byun *et al*., 2001), and desmin (Chen *et al*., 2003) by caspases is not surprising. The caspase-mediated cleavage of IF proteins seems in fact influential for the progress of apoptosis as caspase-resistant vimentin and desmin attenuate programmed cell death (Belichenko *et al*., 2001, Panagopoulou *et al*., 2008). Interestingly, IFs have been established to provide an additional regulatory mechanism for caspase activation. The interaction between K8/K18 and death effector domain containing DNA binding protein (DEDD) creates a platform for caspase-3, thus bringing caspase-3 into close proximity of K8/K18-associated caspase-9 and facilitating the caspase activation (Lee *et al*., 2002, Dinsdale *et al*., 2004). As a further support, the interaction between keratin filaments and DEDD has been depicted to correlate with apoptotic sensitivity (Schutte *et al*., 2006). However, the close relationship between IFs and caspase activation does not reflect merely the role of IFs in amplifying the caspase cascade and, thus apoptosis. Keratin inclusions, resulting from the caspase cleavage of K18 and the collapse of K8/K18 filaments at later stages of apoptosis, sequester proapoptotic factors, including active caspases, DEDD, and TRADD (Dinsdale *et al*., 2004). The isolation of the components of apoptotic machinery is likely to modify the apoptotic program. eIF3k, a subunit of eukaryotic translation initiation factor 3 complex, has been observed to interact with K18 and promote the mobilization of active caspase-3 from K8/K18 inclusions by inhibiting the association between K18 and caspase-3 (Lin *et al*., 2008). The depletion of eIF3k attenuates apoptosis in epithelial cells demonstrating the importance of keratin inclusions as regulators of caspase availability (Lin *et al*., 2008). Moreover, K18 as a major caspase-3 substrate has been proposed to reduce the proteolysis of other caspase-3 target proteins, thereby partially explaining the increased susceptibility of K8/K18-deficient tissues to apoptosis (Leifeld *et al*., 2009).

The susceptibility of keratin-deficient cells to death ligand-induced apoptosis is shown in various contexts. The predisposition of K8/K18 to regulate Fas- versus TNF-mediated apoptosis seems highly cell-type specific. Whereas the exquisite respond to Fas is typical for liver with a modulated K8/K18 network (Ku *et al*., 2003, 2007), the significant embryolethality described in certain K8 KO strains is due to TNF-dependent apoptosis triggered in placental trophoblasts (Jaquemar *et al*., 2003). TNF has been implicated in various physiological processes in placenta ranging from the turnover to invasion of trophoblasts (reviewed by Haider and Knöfler, 2009). This emphasizes the need for keratins in responding to TNF-evoked apoptotic challenge during embryogenesis. The increased susceptibility to TNF-induced apoptosis is also detected among keratinocytes bearing a K14 mutation leading to a rare ectodermal dysplasia, Naegeli-Franceschetti-Jadassohn syndrome, characterized by abnormal apoptotic activity in the epidermal cell layer (Lugassy *et al*., 2008).

To conclude, keratin filaments seem to critically modulate death receptor signaling cascades preventing the unwarranted and too intense induction of apoptosis (Figure 4). So far such a comprehensive regulation of the apoptotic machinery has not been published with other cytoplasmic IF proteins. The observations defining IFs as regulators of mitochondria (Milner *et al*., 2000, Tao *et al*., 2009), prompt to investigate also the possible functions of IFs in the intrinsic, mitochondrial pathway of apoptosis. In fact, the reduced capacity of mitochondria to resist increased cellular Ca^{2+} levels in desmin KO

cardiomyocytes can be overcome by the overexpression of antiapoptotic mitochondrial membrane protein Bcl-2 (Weisleder *et al*., 2004). More recently the morphological alterations and disturbed intracellular distribution of mitochondria in keratin-deficient hepatocytes were shown to correlate with increased sensitivity to Ca^{2+}-induced permeability of mitochondria and increased release of cytochrome c (Tao *et al*., 2009). However, it is worth remembering that these features can also arise from the ability of IFs to mechanically stabilize the mitochondrial structure and, thus to regulate the cytochrome c release, initiating the intrinsic apoptotic pathway.

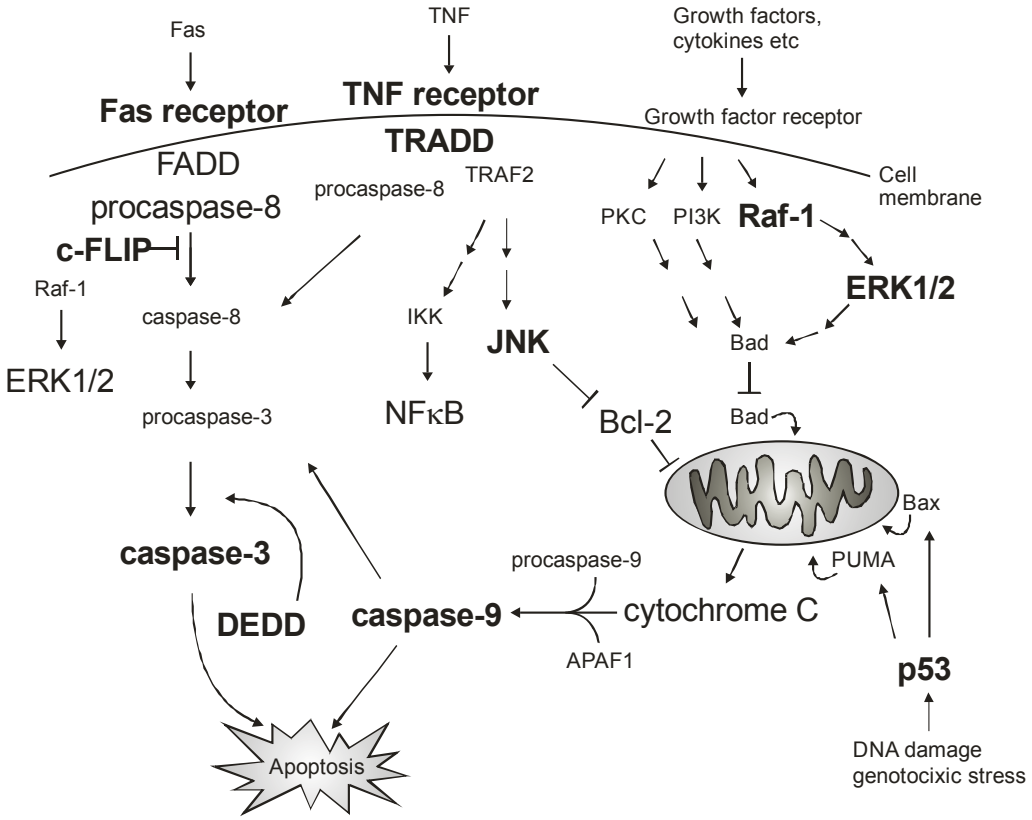

Figure 4. Generalized view on the apoptotic pathways affected by IF-mediated protein sequestration. The association of death ligands to death receptors induces the anchorage of TRADD and FADD to receptors. These adaptor molecules facilitate the binding of procaspase-8, the activation of which induces the caspase cascade. c-FLIP inhibits the procaspase-8 processing, thus impairing the caspase activation, and promotes the antiapoptotic ERK activity through Raf-1. TNF receptor associated factor 2 (TRAF2) mediates through the regulation of IκB kinase (IKK) the activation of NFκB. Same receptor complex promotes the JNK activation which enhances the transcription of proapoptotic genes and inhibits Bcl-2. The members of the Bcl-2 family regulate the intrinsic pathway of apoptosis by controlling mitochondrial permeability. The antiapoptotic Bcl-2 inhibits cytochrome c release whereas the proapoptotic Bad translocating to the mitochondria following death signal promotes the release of cytochrome c. Bad translocation is inhibited by survival factors inducing the phosphorylation of Bad that leads to its cytosolic sequestration. In response to DNA damage, p53 induces the transcription of proapoptotic Bax and PUMA. Upon release from mitochondria, cytochrome c binds to apoptotic protease activating factor 1 (APAF1) forming apoptosome which enables the caspase-9 activation. DEDD creates a platform for caspase-3 facilitating its activation and amplifying caspase cascade. Proteins directly regulated with IFs are written with bigger and bolder font whereas the proteins showing functional consequences upon IF ablation are written with bigger font.

2.1.2 Apoptosis-modulating signaling is affected by intermediate filaments

Although the execution of apoptosis is dependent on the extrinsic receptor-mediated and intrinsic mitochondrial pathways terminating to caspase activation, the apoptotic process is regulated by a wide range of other signaling proteins (Figure 4). The family of mitogen-activated protein kinases (MAPKs) plays an important role in determining the response of cells to apoptotic stimuli, stress, and injuries. K8 has been demonstrated to sequester pro-apoptotic JNK activity upon Fas receptor stimulation (He *et al*., 2002). The interaction with K8/K18 filaments may hold JNK from phosphorylating nuclear targets, such as c-Jun (He *et al*., 2002). The scaffolding properties of IFs have also been linked to the ERK pathway enhancing cell survival. The ability of IF precursors to become transported along MTs is employed in an elaborate manner upon nerve injury, when soluble vimentin subunits enable the transport of phosphorylated ERK (pERK) from the site of axonal lesion to the nerve cell body (Perlson *et al*., 2005). pERK is linked to importin-β and dynein-mediated retrograde transport by means of vimentin that simultaneously sterically protects pERK from dephosphorylation (Perlson *et al*., 2005, 2006). The utilization of vimentin as a long-distance messenger provides an explanation for the specific increase in vimentin abundance upon nerve injury. Recently elevated ERK activity was linked to increased resistance of basal keratinocytes expressing severe EBS mutation to mechanical stress-induced apoptosis in comparison to wild type (WT) keratinocytes (Russell *et al*., 2010). This observation further emphasizes the altered apoptotic response in the presence of disturbed keratin network. In addition, K8 is known to associate with inactive Raf-1, the major upstream regulator of the ERK pathway (Ku *et al*., 2004). Upon stress Raf-1 is released from keratin IFs in association with Raf-1 activation and K18 phosphorylation indicating a keratin-dependent regulatory mechanism for Raf-1 and ERK activities (Ku *et al*., 2004).

Tumor suppressor protein p53 ensures that damaged cells become destroyed by apoptosis by enhancing the expression of proapoptotic genes. The ability of vimentin to associate with p53 (Nishio and Inoue, 2005, Yang *et al*., 2005) represents another paradigm on the involvement of IFs in the regulation of cell death and survival. Treatment with proteasome inhibitor was observed to induce a vimentin-dependent sequestration and accumulation of p53 to the cytoplasm of rheumatoid arthritis synovial fibroblasts (cells causing cartilage destruction in the joints of rheumatoid arthritis patients, Yang *et al*., 2005). This sensitized fibroblasts to TNF-related apoptosis inducing ligand (TRAIL)-induced apoptosis as the caspase-mediated degradation of vimentin caused a nuclear translocation of all the accumulated p53, thus highlighting the potent effect of vimentin on p53.

2.1.3 Intermediate filaments as buffers of cellular stress signals

Extensive work with a variety of mouse and cell culture model systems has proven the essential role of simple epithelial keratins in protecting tissues against various toxin-related stresses. The abundance of keratins, targets of stress-associated kinases, can be utilized during toxin exposure to absorb unwarranted kinase activity underlining the multitude of mechanisms of keratins to regulate stress-induced signal transduction.

K8 and K18 become phosphorylated during different forms of cell stress (Liao *et al*., 1995, 1997, Tao *et al*., 2003). In addition, the hyperphosphorylation of K8/K18 determines the human liver disease progression (Toivola *et al*., 2004b). A clue to the physiological role of stress-mediated K8/K18 phosphorylation was obtained from studies

showing that the mutation of S53 to alanine (S53A; according to the new nomenclature, Omary *et al*., 2009) in K18 eliminating phosphorylation on this particular site predisposes transgenic mice to hepatotoxic injury (Ku *et al*., 1998b). This indicates that keratin phosphorylation could have a protective effect. Although the exact role of stress-induced keratin phosphorylation is not entirely solved the existing theory defines keratin filaments as phosphate sponges or buffers absorbing redundant kinase activity. This is suggested to prevent the stress-associated kinases to regulate the activity of phosphorylation-dependent proapoptotic proteins (Ku *et al*., 2007, Toivola *et al*., 2010). Mutations in K8/K18 have been described to pose a risk for liver diseases with multiple etiologies (reviewed by Omary *et al*., 2002, 2009). The first direct evidence that one of the most common mutations, glycine 62 to cysteine (G62C; according to the new nomenclature, Omary *et al*., 2009) in K8, predisposes to liver damage was got when the transgenic mice overexpressing K8 with this mutation were revealed to be more prone to liver injury and apoptosis than WT mice (Ku and Omary, 2006). Moreover, the G62C-mutation dramatically inhibited K8 phosphorylation at S74, which is known to be targeted by the stress-activated protein kinases. G62C-mutation was suggested to interfere with the ability of S74 to serve as a phosphate substrate, thereby exposing other proteins for increased phosphorylation and, consequently, promoting the progress of apoptosis (Ku and Omary, 2006). The ability to modulate apoptosis by shunting kinase activity may concern several type II keratins. The motif akin to S74 in K8 is conserved in esophageal K4 as well as in epidermal K5 and K6 that equally become phosphorylated during stress (Toivola *et al*., 2002).

Hyperphosphorylation of K8/18 has been observed to mediate the formation of Mallory-Denk bodies (MDBs), cytoplasmic inclusions characteristic to alcoholic and non-alcoholic liver diseases (reviewed by Zatloukal *et al*., 2007, Ku *et al*., 2007, Strnad *et al*., 2008). In addition to hyperphosphorylated keratins, chaperones and sequestosome 1/p62 are the major constituents of MDBs. Despite being prominent morphological features indicating hepatocellular injury, it is still unknown whether the formation of MDBs is deliberate and what the significance of MDBs is. Although MDBs could be expected to disturb protein folding and degradation due to the isolation of respective cellular machineries, MDBs seem not to have any apparent toxic effect and disappear upon recovery when keratin network reassembles. Hence, it has been postulated that MDBs could protect hepatocytes by sequestering toxic and harmful compounds (Zatloukal *et al*., 2007, Ku *et al*., 2007). Considering the effect of apoptotic keratin inclusions to caspase availability, IF inclusions could have specific, sequestration-based functions rather than being mere bystanders. Furthermore, some unexpected pieces of work have revealed increased resistance of cells bearing inclusion-promoting mutations in their IF genes to apoptosis compared to WT cells (Schietke *et al*., 2006, Russell *et al*., 2010). How IF inclusions affect death and survival signaling is among the IF-related questions to be answered.

In summary, IFs act as survival-promoting cell elements regulating various steps in apoptotic signal transduction through protein sequestration and attenuation of the stress-induced kinase activity (Figure 4). Further supporting the antiapoptotic function of IFs, the ablation of IF network often coincides with increased apoptotic sensitivity. Considering that also mechanical forces applied to either epithelial, endothelial (vascular flow) or myogenic cells (contraction) can stimulate apoptotic signals, it remains obscure whether the non-mechanical functions were the primary functions of IFs or arose as a secondary effect of mechanical resistance (Pekny and Lane, 2007).

2.2 Impact of intermediate filaments on tissue growth and regeneration

Although the stress-related features of IFs are extensively studied, substantial amount of research has focused on describing the IF functions in cell growth and proliferation. The ablation of IF proteins has been presented to modulate the proliferation rate of cells both positively (Paramio *et al*., 2001, Reichelt and Magin, 2002) and negatively (Pan *et al*., 2008) elucidating the ability of IFs to regulate the growth-related signaling cascades.

K10, the major type I keratin in postmitotic suprabasal keratinocytes, has been proposed to have antiproliferative features as the expression of K10 inhibits cell growth in a variety of epithelial cell lines (Paramio *et al*., 2001). The negative impact on cell cycle progression seems to stem from the deregulation of phosphatidylinositol 3-kinase (PI3K) pathway through a direct sequestration of Akt and atypical protein kinase C ζ (PKCζ) by the K10 N-terminus (Paramio *et al*., 2001). Mice expressing K10 in basal keratinocytes under the promoter of K5 are characterized by impaired Akt and PKCζ activities in skin, decreased epidermal proliferation and reduced probability to skin carcinogenesis (Santos *et al*., 2002). Interestingly, the ablation of K10 in mice induces hyperproliferation in basal cells expressing K5/K14 but not in suprabasal keratinocytes (Reichelt and Magin, 2002). Instead of Akt, increased MAPK activity observed in K10 KO epidermis was suggested to explain the induction of proliferation (Reichelt *et al*., 2004). Against what could be expected, the papilloma formation was decreased in K10 KO mice, probably due to accelerated cell turnover in K10 KO epidermis (Reichelt *et al*., 2004). These results indicate that a multitude of factors affect the outcome of K10 modulation *in vivo*. Increased Akt activation has also been detected in K8 KO hepatocytes along with more efficient G1/S transition (Galarneau *et al*., 2007). However, rather than a keratin-dependent regulation of Akt, this was estimated to reside from the K8/18-mediated regulation of receptor for activated C kinase 1 (RACK1)/plectin-complex acting as a platform for growth signals, although no direct interaction between K8/18 and RACK1/plectin-complex was presented.

14-3-3 proteins are a family of abundant cellular proteins that play key roles in the regulation of central physiological activities, including cell growth and proliferation, through interactions with a number of signaling determinants (Figure 5; reviewed by Bridges and Moorhead, 2005, Mhawech, 2005, Hermeking and Benzinger, 2006, Tzivion *et al*., 2006). IFs have been illustrated important regulators of 14-3-3. Phosphorylated vimentin sequesters 14-3-3 from another 14-3-3 binding protein Raf whose catalytic activity is dependent on 14-3-3 interaction (Tzivion *et al*., 2000). Phosphorylated K18 interacts with 14-3-3 during the G2/S/M phases of the cell cycle (Liao and Omary, 1996). The N-terminal S34 of K18 (according to the new nomenclature, Omary *et al*., 2009) has been demonstrated to mediate the interaction (Ku *et al*., 1998a). Given that 14-3-3 proteins are known to regulate the G2 to M phase checkpoint through their interaction with the phosphatase Cdc25 (Hermeking and Benzinger, 2006), it was hypothesized that keratin network might affect the cell cycle. Indeed the mitotic progression was disturbed in K8 KO hepatocytes, lacking the K8/18 network (Toivola *et al*., 2001), as well as in hepatocytes from a transgenic mouse overexpressing K18 with a mutated 14-3-3 binding site (S34A; Ku *et al*., 2002). 14-3-3ζ displayed nuclear sequestration both in K8 KO liver cells and in K18 S34A transgenic hepatocytes, compared to its normally rather diffuse cytoplasmic localization, thus indicating that IFs are necessary to control the subcellular localization of 14-3-3 and prevent the uncontrolled and harmful interactions between 14-3-3 and Cdc25 in the nucleus (Toivola *et al*., 2001, Ku *et al*., 2002).

In addition to cell proliferation, IF/14-3-3 interactions have been implicated in determining cell size. 14-3-3σ, an epithelial isoform, associates with K17 in a phosphorylation-dependent manner (Kim *et al*., 2006). The expression of K17 is rapidly increased in epithelial cells at the skin wound edges. Mouse skin keratinocytes lacking K17 are depicted with smaller size than WT keratinocytes, depressed protein translation and delayed wound closure (Kim *et al*., 2006). These defects have turned out to be due to disturbed anchoring of 14-3-3σ in the cytoplasm, where it is needed to stimulate the mammalian target of rapamycin (mTOR) signaling pathway controlling protein synthesis and cell growth, hence pointing to the demand of K17/14-3-3 interaction during regeneration (Kim *et al*., 2006). Phosphorylation of T9 and S44 in the N-terminal domain of K17 was delineated to mediate this interaction (Kim *et al*., 2006). More recently mTOR signaling defects were observed in mouse embryos where the entire type II keratin cluster had been deleted (Vijayaraj *et al*., 2009). These embryos, dying at embryonic day 9.5 (E9.5) because of severe growth retardation, suffer mislocalization of glucose transporters GLUT1 and GLUT3 at the apical membrane of the yolk sac, increased activation of mTOR inhibiting AMP kinase due to limited glucose and, hence, reduced mTOR activity and protein synthesis. The authors proposed a role for keratin IFs in the regulation of glucose availability and cell growth prior to the formation of placenta when the embryo is still dependent on the yolk sac (Vijayaraj *et al*., 2009).

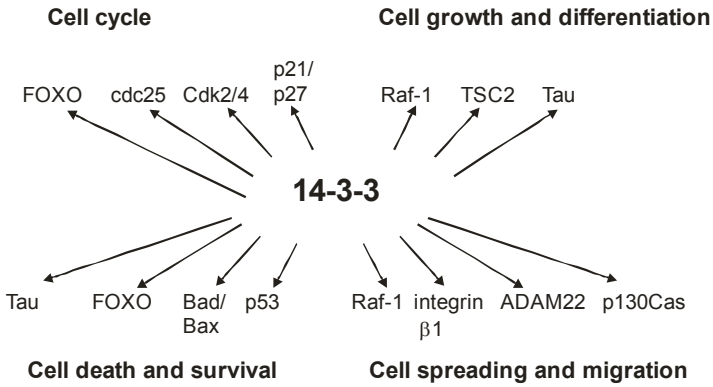

Figure 5. Simplified scheme of cellular events controlled by 14-3-3 with examples of 14-3-3-regulated proteins. FOXO, Forkhead box protein O; p130Cas, p130 Crk-associated substrate; TSC2, Tuberous sclerosis protein 2 (Bridges and Moorhead, 2005, Mhawech, 2005, Hermeking and Benzinger, 2006, Tzivion *et al*., 2006.)

Similarly to the expression of K17 depicting skin injuries, the specific upregulation of GFAP and vimentin is a hallmark of reactive astrocytes in CNS injuries. The ablation of these IF proteins leads to attenuated reactive gliosis (astrocyte activation) at the initial stages of neurotrauma although the later posttraumatic regeneration is improved suggesting that reducing reactive gliosis may actually have profound effects (Wilhelmsson *et al*., 2004, Sihlbom *et al*., 2007). It is noteworthy that the expression of 14-3-3ε is induced in reactive gliosis and is less pronounced in GFAP/vimentin-depleted astrocytes (Sihlbom *et al*., 2007). Although 14-3-3 has been established to associate with GFAP and vimentin in astrocytes (Satoh *et al*., 2004, Li *et al*., 2006), it remains to be elucidated whether the reduced 14-3-3ε protein levels are directly due to deficiencies

in the IF network. Moreover, no apparent changes in cell proliferation in response to neuronal injury was detected in GFAP/vimentin KO reactive astrocytes (Pekny *et al*., 1999), but instead a reduced hypertrophy of cellular processes in individual GFAP/vimentin KO astrocytes after CNS trauma was evident (Wilhelmsson *et al*., 2004). Whether this reflects a link between 14-3-3 and cell growth is still unanswered. The symptoms of GFAP/vimentin KO mice upon nerve injury could also be explained by the changes in endothelin-1 signaling. Endothelin-1, a peptide mediating the vascular tone, has been perceived as a potent regulator of reactive gliosis acting through endothelin B receptor (ET_BR; Gadea *et al*., 2008). The remarkable colocalization between GFAP/vimentin filaments and ET_BR in astrocytes and the disturbed distribution of ET_BR in GFAP/vimentin double KO reactive astrocytes in brain ischemia suggest a direct, IF-dependent regulation of ET_BR (Li *et al*., 2008). Endothelins are known to inhibit the astrocyte gap-junctional communication (AGJC). A less efficient AGJC block indicating enhanced distribution of signals over long distances was observed in IF-deficient astrocytes raising the possibility that disturbed endothelin signaling could be the explanation for the more prominent response to CNS trauma and increased infarct size in GFAP/vimentin KO mice (Li *et al*., 2008).

The importance of glial GFAP has been discerned also in the injuries of peripheral axons. Delayed nerve regeneration in GFAP KO animals arises from the reduced proliferation of Schwann cells, glia of the PNS which normally mediate the axonal regrowth (Triolo *et al*., 2006). GFAP and vimentin were observed to interact with integrin $\alpha v\beta 8$ and $\alpha 5\beta 1$ respectively and, moreover, GFAP was discovered to be an important regulator of fibrin-$\alpha v\beta 8$-ERK1/2 pathway controlling Schwann cell proliferation after injury (Triolo *et al*., 2006). Thus, the mechanisms of IF-mediated regulation of cell growth and proliferation appear highly cell type and context-dependent. Moreover, the ability of K18 to attenuate estrogen receptor α (ERα)-mediated signaling through the anchorage of LPR16, a protein interacting with and regulating the transcriptional activity of ERα, and, thereby to inhibit the estrogen-stimulated cell cycle progression (Meng *et al*., 2009), further highlights the versatile mechanisms of IFs to regulate cell proliferation and tissue growth through protein scaffolding.

2.3 Intermediate filaments contribute to cell homeostasis through protein targeting

Apart from being essential for stress tolerance and tissue growth, IFs have been depicted important in maintaining the cellular homeostasis. Through protein targeting and sequestration IFs regulate cell polarity, cell membrane complexes taking care of cellular nutrition as well as vesicular trafficking affecting the cell membrane composition.

Tissue homeostasis is dependent on epithelial polarization since the correct localization of various proteins/protein complexes either to the apical or basal compartment is critical for their functionality. Prominent apical distribution of keratins in single-layered epithelium and keratin-dependent protein sequestration have been illustrated important for the establishment and maintenance of epithelial polarity (Oriolo *et al*., 2007b). Keratin-mediated scaffolding of a component of γ-tubulin ring complex, GCP6, a protein functioning at MTOCs, improves the enrichment of MTOCs to the apical domain (Oriolo *et al*., 2007a). This is essential for the capping of MT minus-ends directed towards the apical part of the cell and the stabilization of MT network which by providing a

mechanism for protein transport governs an important aspect of epithelial polarization. Furthermore, apical keratin filaments form a ternary complex with the chaperone Hsp70 and the regulator of cellular asymmetry, atypical PKCι (Mashukova *et al*., 2009). Keratin scaffold was suggested to be essential for both the chaperone-mediated rescue of inactive PKCι from proteosomal degradation and the phosphorylation-dependent reactivation of PKCι. This mechanism would maintain the appropriate levels of functional PKCι in epithelial cells (Mashukova *et al*., 2009). Albatross is a protein located in apical junctional complexes which separate the apical and lateral membranes of epithelial cells, thereby preventing mislocalization of membrane proteins and assisting cell polarization. K8/18 filaments have been shown to promote the formation of apical junctional complexes by sequestering and stabilizing Albatross protein (Sugimoto *et al*., 2008).

In addition to the establishment of epithelial polarity, IFs have been linked to the functionality of diverse cell membrane proteins. This function of IFs is well reflected by the disturbed signal transduction originating at the β-adrenergic receptors (βARs) and reduced lipolysis in vimentin-depleted adipocytes (Kumar *et al*., 2007). In response to catecholamine stimulation, βARs recruit Src kinase to stimulate the ERK cascade which leads to the release of stored fatty acids. Vimentin is proposed to constitute a regulatory structure at the receptor enabling efficient signal transmission (Kumar *et al*., 2007). Similarly the reduction of sodium-glucose cotransporter SGLT1 in the membrane lipid rafts and the decreased sodium-glucose transport specify vimentin KO renal proximal tubular cells (Runembert *et al*., 2002). A significant colocalization of vimentin and SGLT1 on the membrane of WT tubular cells suggests a vimentin-mediated incorporation of SGLT1 into rafts. The importance of vimentin in the sodium-glucose transport was highlighted when the specific vimentin re-expression in proximal tubular cells upon renal ischemia was shown to be essential for the localization of SGLT1 and the restoration of sodium-glucose cotransporter activity and, hence the prevention of glucosuria (Runembert *et al*., 2004). Furthermore, severe problems in electrolyte transport have been detected in the distal colon of K8 KO mice suffering colitis and chronic diarrhea (Toivola *et al*., 2004a). Altered localization and impaired function of ion transporters observed in K8 KO colon renders IFs as important regulators of the internal ion equilibrium, although no direct association between the ion transporters and keratins was detected (Toivola *et al*., 2004a).

Endo-lysosomal sorting machinery is critical for the determination of protein content of the cell membrane but participates also in the maintenance of cell homeostasis through the regulation of protein degradation. IFs have been proven to modulate the vesicular transport machinery, thereby affecting the distribution and degradation of proteins. AP-3 is an adaptor protein complex regulating the packing and coating of vesicles delivering proteins from endosomes to late endosomes and lysosomes. Vimentin regulates the distribution of AP-3 and the sorting of the AP-3 binding proteins (Styers *et al*., 2004). Desmin has been demonstrated to bind through myospryn to the biogenesis of lysosome-related organelles complex 1 (BLOC-1) which, according to its name, regulates the biogenesis of lysosome-related organelles such as melanosomes (Kouloumenta *et al*., 2007). The N-terminal domain of desmin is responsible for myospryn sequestration and, therefore, in desmin KO neonatal cardiomyocytes myospryn is diffused throughout the cytoplasm contrary to the normal perinuclear localization. Due to the colocalization of myospryn with lysosomal markers in WT cardiomyocytes and the juxtanuclear aggregation of lysosomes in desmin KO cells, the authors rationalized that desmin-mediated regulation of myospryn and BLOC-1 could

enable the biogenesis and positioning of lysosomes and lysosome-related organelles (Kouloumenta *et al*., 2007). Furthermore, vimentin serves as a reservoir for the synaptosome-associated protein SNAP23, a member of SNAP receptor (SNARE) protein family (Faigle *et al*., 2000). SNAP23 participates in the formation of SNARE complex mediating cell membrane fusion upon exocytosis. In addition, evidence has been presented that the ablation of GFAP and vimentin in astrocytes affects the directional mobility and trafficking of vesicles (Potokar *et al*., 2007, 2010). This indicates that IFs could have profound influence on the storage and release of various neuroactive compounds from astrocytes. Taken together, the scaffolding function of IFs appears critical for the proper subcellular localization of a variety of proteins. In addition, IFs seem to have a pronounced control over the vesicular trafficking system, affecting cell and tissue homeostasis in many ways.

2.4 Nuclear signaling functions of intermediate filaments

Among the most important cellular activities is protein synthesis. In addition to the keratin-mediated regulation of mTOR pathway, affecting the activities of S6 protein kinase and eukaryotic initiation factor 4E, and the ability of epithelial keratins to regulate translation by binding to eukaryotic elongation factor-1 subunit γ (Kim *et al*., 2007), IFs display specific control over nuclear activities including gene transcription. Lamins, IFs of the NE, have been elucidated to function as scaffolds for a variety of nuclear proteins and, in addition, to regulate chromatin organization (Figure 6; reviewed by Dechat *et al*., 2008, Andrés and González, 2009).

Nuclear lamins have been shown to interact with chromatin either directly or indirectly through various lamin-binding proteins. The importance of lamins in chromatin regulation is reflected by disturbances in heterochromatin organization and epigenetic changes in heterochromatin due to modulated histone methylation in lamin-deficient or mutated cells (Dechat *et al*., 2008). Although the details require further dissecting, this function of lamins has been postulated to explain some aspects of laminopathies, a broad range of diseases leading either to myodystrophy, lipodystrophy, neurodystrophy or premature aging (Dechat *et al*., 2008). Studies with amphiregulin (AR), a member of epidermal growth factor (EGF) family, provide an example of lamin-mediated protein sequestration affecting chromatin (Isokane *et al*., 2008). AR is synthesized as a transmembrane protein (pro-AR) the transactivation of which requires ectodomain shedding. The induced transactivation is partial and the remaining pro-AR is endocytosed, proteolytically processed and translocated to the inner NE where it becomes anchored to A-type lamins. Targeting of pro-AR to the NE induces heterochromatin assembly and suppresses the gene expression (Isokane *et al*., 2008). More recently A-type lamins were suggested to repress transcription when anchored to the close proximity of gene promoters probably due to the relocalization of promoters to nuclear periphery, area depicted with heterochromatin and reduced gene activity (Lee et al., 2009). B-type lamins have been detected to tether testis-specific gene clusters to the NE and repress the transcription of genes in these clusters (Shevelyov *et al*., 2009). The derepression of clusters is associated with their detachment from the NE, hence indicating that lamins provide a mechanism to silence multigenic regions (Shevelyov *et al*., 2009). However, the model of repressed gene expression at the nuclear periphery has been criticized to be oversimplified as also opposite results have been presented (reviewed by Takizawa *et al*., 2008). Microenvironment at the NE has been stated to play a key role in gene

silencing but the molecular mechanisms of reduced transcription at the nuclear periphery still remain elusive (Takizawa *et al*., 2008).

Several studies have implicated lamins in the regulation of transcription factors, cell cycle regulator retinoblastoma protein (Rb) being one of the most studied. The nucleoplasmic complexes of lamin A/C and lamin-binding protein LAP2α promote the nuclear localization of Rb through sequestration (Markiewicz *et al*., 2002). Loss of these complexes leads to hyperphosphorylation of Rb, dissociation of transcription factor E2F from Rb, increased expression of E2F target genes and inefficient cell cycle arrest (Naetar *et al*., 2008). Indeed, lamins seem essential for the regulation of Rb phosphorylation as the lamin A/C scaffold has been proposed to facilitate the interaction between protein phosphatase 2A (PP2A) and Rb (Van Berlo *et al*., 2005). The activation of PP2A in response to transforming growth factor-β1 (TGF-β1) signaling is followed by the dephosphorylation of Rb. Thus, lamins appear to enable an important switch in the regulation of cell proliferation and tissue turnover. Furthermore, lamins have been shown to protect Rb from proteosomal degradation (Johnson *et al*., 2004).

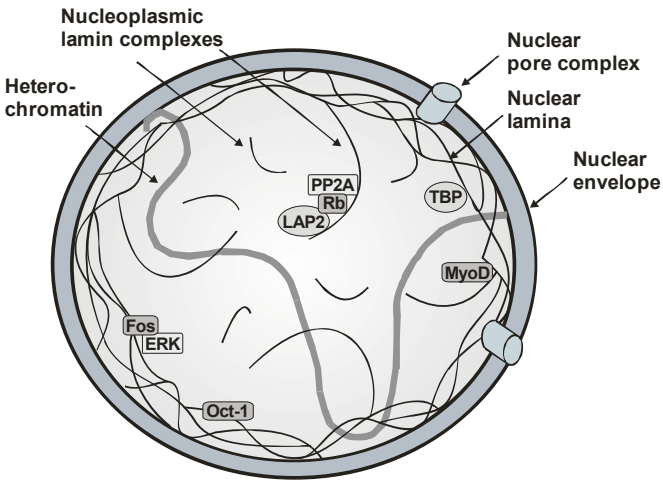

Figure 6. Schematic picture of the organization of nuclear lamina and the scaffolding functions of lamins. Filamentous layer of nuclear lamins associates with the inner NE. In addition, lamins can be found in throughout the nucleoplasm. Lamins regulate heterochromatin and a broad range of transcription factors through their scaffolding ability. Examples of lamin-interacting proteins are covered in the text.

Rb is not the only proliferation-related transcription factor regulated by lamins. Type A lamins have been elucidated to affect Activator protein-1 (AP-1) dependent transcription by sequestering c-Fos to the NE through a direct interaction with bZIP region of c-Fos (Ivorra *et al*., 2006). bZIP is essential for c-Fos heterodimerization with other AP-1 family members, such as c-Jun, and, thus for AP-1 activity. In fact, ectopic expression of lamin A induces growth arrest (Ivorra *et al*., 2006). Later on the lamin A scaffold has been found to mediate the mitogen-induced AP-1 activation. Activated ERK1/2 interacts with lamin A and phosphorylates c-Fos which subsequently becomes released from the NE allowing the rapid transcription of AP-1 regulated genes (González *et al*., 2008). The regulation of transcription concerns also B-type lamins. Octamer transcription factor 1 (Oct-1) modulates both developmental processes and stress response. Lamin B1-dependent sequestration of Oct-1 to the nuclear periphery has turned out significant for gene expression and resistance to oxidative stress (Malhas *et al*., 2009). This interaction may also play a role in aging as reactive oxygen species (ROS) are involved in aging process (Malhas *et al*., 2009).

The initiation of A-type lamin expression correlates with the progress of differentiation. Intriguingly, several muscular dystrophies are known to reside from mutations in the *LMNA* gene (Worman and Bonne, 2007). The ablation of lamin A in myoblasts impairs the exit of myoblasts from the cell cycle upon mitogen withdrawal and hampers myogenic differentiation, suggesting that compromised muscular regeneration would contribute to the generation of muscular dystrophies (Frock *et al*., 2006). MyoD, a key regulator of muscle differentiation, has been implicated as a lamin A binding protein (Bakay *et al*., 2006). During myogenesis, hypophosphorylated Rb recruits histone deacetylase 1 (HDAC1) from MyoD, the activity of which is promoted by acetylation (Puri *et al*., 2001). Based on a microarray analysis of muscle biopsies it was suggested that these key signaling events of myogenic differentiation leading to MyoD acetylation would occur on the lamin scaffold, hence providing a molecular mechanism for the defects in lamin A-depleted muscle (Bakay *et al*., 2006). In addition to myogenic factors, an interaction between lamin A and an adipocyte transcription factor sterol response element binding protein 1 has been described (Lloyd *et al*., 2002). Deregulation of this interaction may have profound effect on laminopathies affecting the subcutaneous adipose tissue (Maraldi *et al*., 2008).

Nuclear lamina regulates also gene activity by affecting the transcriptional machinery. The disruption of the lamina by the overexpression of mutated lamin is associated with the accumulation of TATA binding protein (TBP) to lamin aggregates and to reduced transcriptional activity of RNA polymerase II indicating that lamins can serve as a scaffold for transcription complex (Spann *et al*., 2002). In summary, lamins can be stated to function as a docking platform for nuclear proteins (Figure 6), thus providing a mechanism to regulate nuclear signals and gene expression. Furthermore, the lamin-binding NE proteins interacting with transcriptional regulators further expand the network of nuclear signals affected by the lamina (Andrés and González, 2009).

2.5 Intermediate filaments as mediators of cell motility

It is well established that IFs regulate cell migration and it is becoming increasingly evident that the ability of IFs to function as protein platforms is utilized in determining the motile and adhesive properties of cells. Migrational defects observed in vimentin KO animals during wound healing (Eckes *et al*., 1998, 2000) and the numerous examples illustrating the concurrence of enhanced migration and vimentin expression (for example Mendez *et al*., 2010) render vimentin the most studied IF protein in terms of migration. Vimentin has been described to accumulate to focal adhesions, associate with integrins and form structures sharing features of both actin-tethering focal adhesions and keratin-containing hemidesmosomes termed vimentin associated matrix adhesions (reviewed by Ivaska *et al*., 2007). Vimentin seems to critically regulate the size and distribution of adhesion sites (Tsuruta and Jones, 2003) and the strength of cell adhesion (Bhattacharya *et al*., 2009). This is exemplified by the reduced ability of vimentin-depleted endothelial cells to resist flow-induced mechanical forces due to their inability to maintain the adhesion to substratum (Tsuruta and Jones, 2003).

Vimentin associates with cell adhesion receptors, integrins, plectin being depicted to function as a linker protein (Gonzales *et al*., 2001, Homan *et al*., 2002, Kreis *et al*., 2005). The interplay between vimentin and integrins is bidirectional. Integrin β3 has been presented to regulate the recruitment of vimentin to focal adhesion, thus ensuring the adequate strength of adhesion (Bhattacharya *et al*., 2009), whereas vimentin has been

shown to modulate the integrin content on the cell membrane. Integrin internalization followed by subsequent recycling back to the plasma membrane is an important mechanism for cells to regulate the speed and directionality of migration (Caswell and Norman, 2006). Vimentin is demonstrated to associate with integrin β1 containing vesicles (Ivaska *et al*., 2005). This interaction is released by the PKCε-mediated phosphorylation of vimentin head domain that enables the recycling of the endocytosed integrin β1 to the membrane and promotes cell motility. An actin cross-linking protein filamin A is suggested to co-operate with vimentin in integrin β1 trafficking during cell adhesion and spreading (Kim H *et al*., 2010a, 2010b). Filamin A interacts with PKCε and vimentin and mediates the PKCε-dependent vimentin phosphorylation and, thereby the cell membrane translocation of integrin β1. The importance of vimentin in the regulation of adhesion molecules is further highlighted by a report showing that vimentin IFs are crucial for the attachment of lymphocytes to the vascular endothelium and the transcellular migration of lymphocytes through endothelial cells (Nieminen *et al*., 2006). The perturbations observed with vimentin KO mice were presented to stem from the decreased amounts of integrin β1 on lymphocytes and intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 on endothelial cells. Furthermore, the clear leakiness of vimentin KO endothelium (Nieminen *et al*., 2006) may reflect impaired integrin-cell matrix binding known to induce microvessel leakiness (Wu, 2005). The role of vimentin in integrin downstream signaling has not been addressed although the adaptor protein p130Cas serving as a protein platform at focal adhesions associates with vimentin network (Wang *et al*., 2007). Furthermore, an important organizer of focal adhesions, Rho-kinase, is sequestered by vimentin filaments (Sin *et al*., 1998). Vimentin phosphorylation by Rho-kinase and subsequent IF collapse is associated with the release of Rho-kinase from vimentin and translocation to the cell periphery. This implies that vimentin scaffold could participate also to the transport of adhesion-related signals originating at the cell membrane.

Anterior-posterior polarization is an important phenomenon occurring in migrating cells. Vimentin has been implicated as a regulator of Scrib, a protein linked to polarized migration (Phua *et al*., 2009). In addition to prominent interaction, vimentin was detected to protect Scrib from proteosomal degradation. Importantly, the downregulation of vimentin and Scrib led to very similar disturbances in directed migration and cell-cell adhesion, thereby suggesting that IF-Scrib association is a prerequisite for the function of Scrib in adhesion and migration (Phua *et al*., 2009).

Although extensively studied, vimentin is not the only IF protein linked to cell adhesion and migration. Synemin localizes to the leading edge of migrating cells and affects the MF dynamics (Uyama *et al*., 2006, Pan *et al*., 2008). Indeed, synemin was recently reported to interact with zyxin, focal adhesion associated protein affecting MF assembly and the migratory properties of cells (Sun *et al*., 2010). K6/K16/K17 become expressed upon epidermal injuries at the wound edge and are involved in the healing process, dependent on rapid mobilization and increased migration of keratinocytes (Mazzalupo et al., 2003, Wong and Coulombe, 2003). K8 KO hepatocytes have been demonstrated to exhibit altered adhesion and spreading properties compared to WT hepatocytes (Galarneau *et al*., 2007). This was suggested to be due to the increased density of integrin β1 at the cell membrane of K8 KO hepatocytes. Furthermore, the more rapid adhesion and impaired spreading of K8 KO cells on fibronectin were associated with modulations in the regulation of focal adhesion kinase (FAK), a key protein mediating integrin-induced cellular signals, and Akt (Galarneau *et al*., 2007). These results indicate

that keratins can have a control over integrins as well as the downstream effects of integrin ligation.

3 Intermediate filament protein nestin

The IF protein nestin was identified in 1990 when Lendahl and colleagues were searching for the protein recognized by the antibody Rat.401, specifically targeting the proliferative cells in developing neural tube. They named their discovery according to its expression to neuroepithelial stem cell protein, nestin. With the proteome information from developing CNS Lendahl and colleagues (1990) wished to shed light on the molecular mechanisms of stem cell differentiation into neurons and glia. During the past 20 years nestin has been widely used as a marker for the stem and progenitor cells of various tissues but its functional importance is only recently starting to become evident.

3.1 Expression of nestin

3.1.1 Nestin in developing and regenerating central nervous system

The expression of nestin in developing CNS follows a strict temporal pattern since the expression appearing as early as E7-E8 in the emerging neural tube is rapidly declining when development proceeds (Dahlstrand *et al*., 1995). In rat cerebrum, representing an early developing part of brain, nestin expression peaks at E16 whereas in later developing cerebellum the amount of nestin mRNA is at the highest at postnatal day 5 (P5; Lendahl *et al*., 1990). By P9 the expression of nestin is downregulated in both compartments. In addition, nestin expression determines the neuronal and glial progenitor cells in sympathetic ganglia of mouse PNS where the expression of nestin is at the highest at P1 and becomes gradually downregulated by P18 (Shi *et al*., 2008). In the adult brain nestin defines the neuronal stem cells in subventricular and subgranular zones (Lagace *et al*., 2007). In mature neurons nestin is displaced by NFs and peripherin whereas in mature glia cells GFAP is the major IF protein. Specific upregulation of nestin expression occurs upon regeneration of injured CNS. Nestin expression increases in reactive astrocytes forming a glial scar shortly after the spinal cord or optic nerve injuries in rats (Frisén *et al*., 1995). Similarly, the optic nerve lesion in mouse eye increases nestin expression in astrocytes (Wohl *et al*., 2009). In addition, increased nestin expression correlates with increased proliferation in the progenitor cells of hippocampal subgranular zone following cerebral ischemia (He *et al*., 2005). Detection of nestin in the temporal neocortex of patients with intractable epilepsy equally indicates accelerated gliosis and neurogenesis (Wang et al., 2009). Taken together, the expression of nestin defines both the developing and regenerating CNS.

3.1.2 General mechanisms of myogenesis – nestin in myogenic tissue

Skeletal muscles emerge from the paraxial mesoderm during early embryogenesis. Paraxial mesoderm first separates into segments, cell clusters called somites. The somites are further specialized when cells that retain the expression of transcription factors Pax3 and Pax7 form the dermomyotome. An important step of muscle development, the generation of myotome, is initiated when progenitor cells determined to myotome precursors migrate beneath the dermomyotome. These myotome precursor

cells become myoblasts while they downregulate Pax3 and upregulate myogenic regulatory factors (MRFs). Myoblasts are withdrawn from the cell cycle and subsequently fuse to form multinucleated myofibers. Pax3 and Pax7 positive cells from the eventually disintegrating dermomyotome migrate to the myotome and proliferate extensively prior to the generation of myofibers, thereby expanding the emerging muscle tissue. A portion of these Pax3 and Pax7 positive cells gives rise to satellite cells responsible for muscle regeneration in adult tissue (reviewed by Francis-West *et al*., 2003, Shi and Garry, 2006, Grefte *et al*., 2007).

The regulatory cascade directing myogenesis is composed of MRFs (Figure 7). This family of basic helix-loop-helix transcription factors, including MyoD, Myf5, myogenin and Mrf4, plays a central role in myogenesis (Shi and Garry, 2006). MyoD, Myf5 and Mrf4 are required by the precursor cells to adapt the myogenic fate whereas MyoD, myogenin and Mrf4 determine the terminal differentiation. In addition, the myocyte-specific enhancer factor 2 (MEF2) regulatory factors, belonging to the MADS-box family of transcription factors, are depicted crucial for muscle generation and coordinate gene expression at all stages of development (Sandmann *et al*., 2006). Paired-box transcription factors Pax3 and Pax7 seem equally essential for myogenesis. They are required for the delamination and migration of myogenic progenitor cells from the dermomyotome as well as to the entry of progenitor cells to myogenic program (reviewed by Buckingham and Relaix, 2007). Furthermore, Pax deficiency compromises cell proliferation and survival. The permanent cell cycle withdrawal of myoblasts committed to differentiate is accomplished with cyclin-dependent kinase inhibitors (CKIs) p18, p21 and p27 (Myers *et al*., 2004). In addition, secreted signaling molecules, such as Wnt, Notch and Sonic hedgehog, are known to be involved in muscle development (Francis-West *et al*., 2003, Buckingham, 2006).

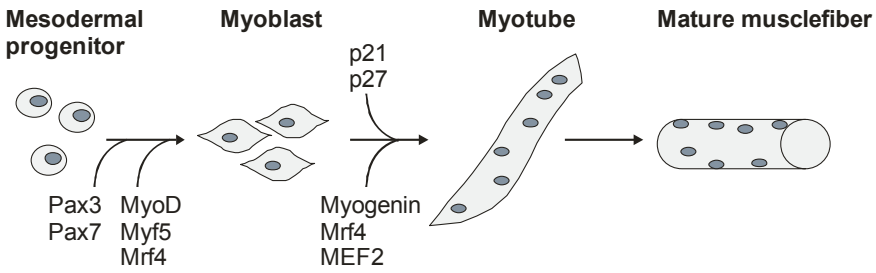

Figure 7. Simplified schematic presentation of myogenic differentiation. Mesodermal progenitor cells are committed to the myogenic lineage. Myoblasts fuse to multinucleated myotubes which then mature to myofibers. The progress of differentiation is critically regulated by the presented regulatory factors.

An important feature of adult skeletal muscle is the ability to self-regenerate following an injury (reviewed by Shi and Garry, 2006, Grefte *et al*., 2007). New skeletal muscle emerges from myogenic precursor cells called satellite cells residing beneath the basement membrane of muscle fibers. The expression of different growth factors is enhanced at the site of injury leading to the activation of quiescent satellite cells and their transformation into myoblasts. The myoblasts proliferate and upregulate MRFs. Following the downregulation of Pax3 and Pax7 myoblasts are committed to differentiate and either fuse to the damaged myofibers or fuse to form new multinucleated myofibers,

thereby repairing the injured muscle. An essential feature of satellite cells is the capability of self-renewal which maintains the number of satellite cells during the rounds of regeneration and normal tissue turnover (Shi and Garry, 2006, Grefte *et al*., 2007). During aging, the regeneration is increasingly ineffective and the amount of satellite cells declines due to impaired proliferative capacity.

Nestin is expressed in developing skeletal (Sejersen and Lendahl, 1993) and cardiac muscle (Kachinsky *et al*., 1995). In the developing rat limbs nestin can be observed at E11 in mesenchymal cells (Wroblewski *et al*., 1996). When embryo development proceeds to E15-E18 the expression of nestin is concentrated to muscles (Sejersen and Lendahl, 1993, Wroblewski *et al*., 1996) to become downregulated by P21 (Sejersen and Lendahl, 1993). In embryonic mouse cardiomyocytes the expression of nestin is extremely transient and can be detected only at E9-E10 (Kachinsky *et al*., 1995). In adult skeletal muscle the overall nestin expression is low except the specific accumulation of nestin to the restricted sarcoplasmic regions, myotendinous and neuromuscular junctions (NMJs; Vaittinen *et al*., 1999). The specific transcription of *nestin* at the NMJs has been confirmed by *in situ* hybridizations (Kang *et al*., 2007).

While comparing muscle biopsies from patients with Duchenne/Becker muscular dystrophy or myositis to biopsies from healthy donors, nestin immunoreactivity was detected in a subset of muscle fibers from all the patients (Sjöberg *et al*., 1994a). Later on it became obvious that nestin, vimentin and desmin are expressed in regenerating skeletal muscle, the expression dynamics resembling closely those observed during muscle differentiation (Vaittinen *et al.*, 2001). This indicates that the differentiation of activated satellite cells proceeds equivalently to embryonic muscle precursors (Vaittinen *et al.*, 2001). As a result of denervation of adult skeletal muscle the expression of nestin increases and nestin becomes spatially distributed from NMJs (Vaittinen *et al*., 1999). During reinnervation nestin distribution is restored implying that the innervation status of muscle fibers determiners the nestin localization and expression in fully developed skeletal muscle (Vaittinen *et al*., 1999). However, in another study the denervation of muscle was associated with the disappearance of nestin immunoreactivity in muscle fiber (Kang *et al*., 2007). Instead, a striking increase in *nestin* transcription in the Schwann cells covering the nerve terminals was observed (Kang *et al*., 2007). In infracted cardiac muscle nestin positive, myocyte-like cells have been detected to encircle the ischemically damaged area (Scobioala *et al*., 2008, Béguin *et al.*, 2009). In a hypothetical model small progeny of nestin expressing cardiac stem cells gives rise to cardiac progenitor cells but whether these cells eventually differentiate with potential to repair the damaged tissue is currently unknown (Scobioala *et al*., 2008, Béguin *et al.*, 2009). Nestin expression is evident also in developing and regenerating vascular smooth muscle cells (Oikawa et al., 2010). To conclude, the expression patterns of nestin in muscle tissue closely resemble its expression in CNS being prominent during both development and regeneration.

3.1.3 Nestin determines the developing and regenerating cells of various tissues

After the initial findings of nestin expression in CNS and muscle, nestin has been shown to be enriched in a multitude of cell and tissue types during development and regeneration. These include developing testis (Fröjdman *et al*., 1997), developing eye (Yang *et al*., 2000), developing and injured tooth (About *et al*., 2000), developing pancreas (Selander and Edlund, 2002), and hair follicle stem cells (Amoh *et al*., 2009). The expression of nestin has also been observed in the endothelial cells of newly formed

blood vessels in developing embryo and regenerating infracted myocardium (Mokrý *et al*., 2004). The latter example, representing neovascularization of postnatal tissue, points out another regenerative process characterized by nestin expression. In addition, endothelial nestin has been depicted in developing kidney, where nestin is transiently expressed in glomerular endothelial cells (Chen *et al*., 2006, Bertelli *et al*., 2007).

Although mostly typifying progenitor cells, nestin expression has been detected to persist in the podocytes of mature glomeruli in adult kidney (Chen *et al*., 2006, Bertelli *et al*., 2007). Moreover, the evident upregulation of nestin is accompanying kidney injuries. Upon puromycin aminonucleoside-induced nephrosis, an experimental podocyte injury model, nestin expression in podocytes increases (Zou *et al*., 2006). An antibody-mediated destruction of the mesangium, the inner layer of glomerulus, represents another kidney injury model. In response to antibody treatment mesangial reserve cells start to proliferate and migrate to repair the destroyed mesangium. *De novo* expression of nestin occurs while mesangial cells move to the proliferative phase and start regeneration (Daniel *et al*., 2008). Nestin is present also in a variety of cell types in adult pancreas although the expression of nestin seems very heterogeneous (Street *et al*., 2004). Whether nestin can be used as marker for adult pancreatic stem cells and whether these cells can be utilized in the production of β-cells destroyed at the pancreatic islets of Langerhans in diabetes has been under investigation for a decade. Recently it was shown that the depletion of nestin in pancreatic duct stem cells hampers their differentiation into insulin producing cells but the mechanism stays unclear (Kim SY *et al*., 2010)

Cancer and progenitor cells share many features including enhanced proliferation, survival and motility. Soon after the initial characterization of nestin in the CNS, nestin was observed to be expressed in brain tumors, especially in gliomas exhibiting malignant behavior (Tohyama *et al*., 1992). It was proposed that nestin, depicting various CNS tumors of neuroectodermal origin, could define the tumors originating from neuronal progenitor cells (Dahlstrand *et al*., 1992a). More recently nestin has been offered as a marker for tumors originating from stem cells (for example Sellheyer *et al*., 2009) although the exact role of these stem cell-like cancer cells in tumor initiation and progression is still under a debate. In addition, nestin has been suggested to serve as a molecular link between tissue regeneration, dependent on the activation of progenitor cells, and cancer risk (Kleeberger *et al*., 2007). Hitherto the expression of nestin has been shown to determine tumors of various origin including gastrointestinal stromal (Tsujimura *et al*., 2001), testicular (Lobo *et al*., 2004), odontogenic (Fujita *et al*., 2006), prostate (Kleeberger *et al*., 2007), pancreatic (Kawamoto *et al*., 2009), thyroid (Yamada *et al*., 2009), and mammary gland tumors (Liu *et al*., 2010).

3.1.4 Regulation of nestin expression

A great amount of work has been aimed at characterizing the factors regulating nestin expression. The second intron of the *nestin* gene is known to be essential for the expression of nestin in CNS whereas the first intron, containing E box motifs possibly recognized by MRFs, regulates nestin expression in muscle precursors (Sejersen and Lendahl, 1993). The transcription factors of SOX and POU families have been indicated important in the production of *nestin* mRNA in developing CNS (Tanaka S *et al*., 2004). Thyroid transcription factor-1, regulating forebrain organogenesis, is another potential candidate for nestin expression (Lonigro *et al*., 2001). In highly malignant neuroblastoma cells N-Myc appears as a putative regulator of nestin expression as it binds to the

second intron (Thomas *et al*., 2004). The second intron of the human and mouse *nestin* genes also contains a binding site for CBF-1, the activity of which is regulated by the Notch signaling (Shih and Holland, 2006). Indeed, in K-ras-induced mouse models of glioblastoma multiforme, the expression of nestin correlates with the Notch activation. Notch signaling also appeared important for the maintenance of nestin immunoreactivity in neuronal stem cell line (Mellodew *et al*., 2004). Following transplantation to the rats undergone striatal lesion or co-culture on primary hippocampal slices, the amount of nestin in these stem cells was rapidly reduced due to proteosomal degradation. The reduction of nestin represented an early event in the progress of neuronal differentiation and was reversed by the ectopic expression of Notch intracellular domain (Mellodew *et al*., 2004). Fairly little data is available considering the regulation of the first intron of *nestin* but in murine systems it has been shown to contain a consensus sequence for Wilms' tumor suppressor 1 (WT1), specifically concentrated to the developing and mature podocytes (Chen *et al*., 2006). However, in another publication showing similar expression patterns for nestin and WT1 in developing kidney, developing epicardium, and coronary vessels, WT1 was suggested to affect through intron 2 (Wagner *et al*., 2006).

Growth factors have been indicated in the regulation of nestin expression in several contexts. An insulin-like growth factor has a promoting effect on nestin expression during myogenesis (Wroblewski *et al*., 1996). In cultured mesangial cells platelet-derived growth factor (PDGF) has been indicated to stimulate nestin expression (Daniel *et al*., 2008). Similarly in cultured vascular smooth muscle cells the expression of nestin is positively regulated by PDGF and EGF the downstream effectors being ERK and Akt (Oikawa *et al*., 2010). In another study the binding of thrombin to G protein coupled receptor (GPCR) induced a transactivation of EGF receptor in vascular smooth muscle cells leading to ERK-dependent nestin production (Huang YL *et al*., 2009a). Interestingly, the expression of thrombin-specific GPCR is upregulated in arteries following balloon injury which could indicate the injury-associated expression of nestin (Huang YL *et al*., 2009a). The positive effect of thrombin to cell proliferation and nestin expression has also been detected in cultures of radial glia isolated from embryonic cerebellum (Wautier *et al*., 2007). In dental pulp cells and odontoblasts bone morphogenic protein 4 seems responsible for nestin expression (About *et al*., 2000).

3.2 Structural features of nestin

Due to the remarkable differences in the *nestin* transcript compared to the gene products of other IF proteins identified by 1990, nestin was categorized as a novel type VI IF protein (Lendahl *et al*., 1990). However, more elaborate characterization of the human *nestin* gene revealed that the intron-exon structure resembled closely NFs (Dahlstrand *et al*., 1992b). Hence, nestin has been later included into the type IV IF proteins. Relatively high variation can be observed when comparing the sequences of the C-terminal tails of human and rodent nestin indicating that the *nestin* gene is undergoing a dynamic evolution (Dahlstrand *et al*., 1992b, Steinert *et al*., 1999, Herrmann *et al*., 2009)

Nestin polymerizes *in vitro* with vimentin and α-internexin (Steinert *et al*., 1999). In vimentin deficient primary astrocytes nestin appears unable to form filaments with residual GFAP further corroborating the prominent predisposition of nestin to assemble into filaments with vimentin (Eliasson *et al*. 1999). In myogenic cells nestin colocalizes with vimentin and desmin filaments (Sjöberg *et al*., 1994b) but the ability to assemble

into filaments with desmin has not been investigated. According to *in vitro* studies, nestin assembles with vimentin at an optimal molar ratio of 1:4 (Steinert *et al*., 1999). Nestin has been proposed to form heterodimers with vimentin and α-internexin but the details of the integration of the heterodimers to the IF network remain to be investigated (Steinert *et al*., 1999). As indicated in section 1.2.2, the short N-terminal domain of nestin (11 amino acids) is the presumable explanation for the inability of nestin to form homopolymers. However, the long, highly charged C-terminal domain of nestin (1300-1600 amino acids) containing glutamate-rich repeats has equally been suggested to interfere the IF assembly (Steinert *et al*., 1999). Indeed, also the C-terminal phosphorylation of nestin on threonine 316 (T316) by Cyclin-dependent kinase 1 (Cdk1) has been associated with the mitotic disassembly of nestin filaments (Sahlgren *et al*., 2001). Question regarding whether this peculiar C-terminal domain of nestin is oriented parallel to filament or protrudes from the IF core, similarly to the C-terminal tails of NFs (Perrot *et al*., 2008), has not been answered yet.

3.3 Functional importance of nestin

At the time nestin was identified, IFs were considered mainly as structural proteins. Hence, nestin was hypothesized to support the progenitor cells encountering structural challenges due to increased proliferation, migration or morphological remodeling occurring during developmental processes such as neurulation (Lendahl *et al*., 1990). This hypothesis was supported by the observations revealing a prominent accumulation of nestin mRNA at the proliferating regions of developing CNS (Dahlstrand *et al*., 1995). Nestin containing IFs were also speculated to possess faster polymerization kinetics suitable for undifferentiated cells in the phase of active proliferation (Sejersen and Lendahl, 1993).

In 2003 Chou and coworkers reported that nestin actually promotes the disassembly of vimentin filaments during mitosis. Transient transfection of nestin caused a formation of non-filamentous vimentin aggregates in mitotic cells where vimentin normally retained its filamentous pattern while reorganizing around the mitotic apparatus. The disassembly of vimentin correlated with the increased phosphorylation of S55 in vimentin head domain indicating that nestin/vimentin heterofilaments could be more prone to phosphorylation (Chou *et al*., 2003). Due to the short N-terminus of nestin, possibly incapable for intrafilament interactions, vimentin modifications could perhaps have a more prominent effect on the filament structure (Chou *et al*., 2003). Thereafter, postulates have been presented that the expression of nestin would render vimentin filaments less stable, thereby alleviating mitosis in fast proliferating endothelial cells (Mokrý *et al*., 2004), mesangial cells (Daniel *et al*., 2008) and EGF-treated vascular smooth muscle cells (Huang YL *et al*., 2009a). Recently the RNAi-mediated downregulation of nestin was reported to suppress the proliferation of neuronal progenitor cells (Xue and Yuan, 2010). The mitotic vimentin/nestin aggregates seem also to have a functional role as they have been detected to sequester insulin-degrading enzyme (IDE) and modulate its activity (Chou *et al*., 2009). It was suggested that nestin would turn vimentin more accessible for IDE by facilitating the IF disassembly and vimentin phosphorylation, critical denominator of the IDE interaction. However, further research is required to clarify the physiological significance of this phenomenon.

Referring to their previous studies with the rapidly moving non-filamentous IF particles (Helfand *et al*., 2003b), Chou and colleagues (2003) presented a hypothesis that the

exceptionally long C-terminal tail of nestin could be utilized in transporting of “cargo” molecules. Asymmetric cell division giving rise to two daughter cells with different properties plays an important role in the development of CNS. Hence, the nestin-mediated filament disassembly and the motility of vimentin/nestin particles could take part into the distribution of cellular factors to daughter cells (Chou *et al*., 2003). Intriguingly, the asymmetric distribution of nestin during the division of neuronal progenitor cells has been reported to regulate cell fate. Prostate apoptosis response-4 (Par-4) positive daughter cells are committed to ceramide-induced apoptosis whereas the nestin positive daughter cells survive to again divide or differentiate (Bieberich *et al*., 2003). This may have profound effects in neuronal stem cell transplantation as the ceramide-treatment of neuronal cultures to be transplanted reduces the number of Par-4-positive cells showing increased tendency towards teratoma formation (Bieberich *et al*., 2004). In mitotic neuroepithelial cells of avian embryos, nestin ortholog transitin regulates the asymmetric distribution of Numb, a regulator of the Notch signaling, through a direct interaction (Wakamatsu *et al*., 2007). Transitin besides segregates but also stabilizes Numb, thereby affecting the proliferation and differentiation of neuroepithelial cells. Due to species-specific differences in the nestin C-terminal domain, however, it remains elusive whether this kind of regulation occurs in human or rodents (Wakamatsu *et al*., 2007). In fact, in mouse cortical progenitor cells derived from E13 cortex undergoing active neurogenesis, Numb was efficiently segregated to the nestin negative cell, although in progenitor cells derived from E10 cortex the divergent distribution of Numb and nestin was not as evident (Shen *et al*., 2002). Recently, nestin-mediated protein sequestration has been linked even more closely to cellular signal transduction. Nestin has been shown to serve as a cytoplasmic anchor to unliganded glucocorticoid receptor (GR; Reimer *et al*., 2009). The interaction between nestin and GR is prominent in embryonic brain as well as in embryo-derived cell cultures and hypothesized as an important regulatory mechanism in embryogenesis as nuclear accumulation of GR enforces cell cycle arrest. Hence, nestin plays an important function as a subcellular organizer affecting critical cell fate decisions.

Although the early theory suggested an important role for nestin in cells undergoing morphological remodeling (Lendahl *et al*., 1990), nestin has not been notably associated with the structural features of cells. However, at the NMJs nestin has been shown to be associated with the junctional elements beneath the postsynaptic receptors (Kang *et al*., 2007). Moreover, nestin filaments are oriented towards the costameres and synaptic nuclei because of which nestin has been assumed to provide mechanical stability and molecular organization for NMJs (Kang *et al*., 2007). In podocytes the downregulation of nestin is associated with the impaired formation of podocyte processes that normally form a network wrapping the capillaries and control the filtration of material from blood to Bowman's capsule as primary urine (Chen *et al*., 2006). As podocytes are presented to have a mechanical role in preventing the outward ballooning of the vessel, nestin network might be important in the maintenance of the functionality of podocytes (Chen *et al*., 2006). Whether nestin works solely on mechanical level stabilizing the podocyte structure or regulates, for example, mechanotransduction requires further studies.

Despite several studies indicating that nestin regulates proliferation during both physiological processes and cancer (Thomas *et al*., 2004), nestin was reported not to affect the growth rate of prostate cancer cells (Kleeberger *et al*., 2007). Therefore, the authors presented that nestin might permit rapid proliferation but would not always be required for it. Instead, they provided evidence that nestin is essential for the migration of prostate cancer cells as well as to the formation of prostate cancer metastases after

xenografting *in vivo*, hence confirming the long-term speculations regarding nestin as a regulator of migration (Kleeberger *et al*., 2007). Moreover, nestin expression has been observed to correlate with lymph node metastasis in a subset of breast cancers not responding to hormonal therapies (Liu *et al*., 2010) and become accumulated in the invasive tumor cells at the periphery of gliomas (Strojnik *et al*., 2007). However, in patients with non-small cell lung cancer nestin expression was associated with lymphangiogenesis which could also explain the increased lymph node metastases and indicate the versatility of nestin-mediated effects in cancer metastasizing (Chen Y *et al*., 2010). Related to the motile properties of cells, a formin-family protein Fhos2 has been proposed to interact with nestin (Kanaya *et al*., 2005). An actin-organizing protein Fhos2 inducing stress fibers, localizes to nestin IFs in embryonic brain and heart but more studies are required to understand the biological importance of this interaction.

In developing cortex, the IFs of neuronal progenitor cells, nestin and vimentin, have been proposed to affect the migration of neuronal progenitors away from the ventricular zone (Miyata and Ogawa, 2007). IFs were suggested to participate in the twisting of the pial processes of neuronal progenitors directed towards the outer surface of developing brain, thereby enabling a spring-like mechanism catapulting the cells away from the ventricular zone. This was dependent on the loss of ventricular contact and the transition of bipolar progenitor cells to unipolar. The involvement of IFs was based on the fact that the treatment with a phosphatase inhibitor known to induce IF disassembly severely impaired the pial processes. This novel mechanism was suggested to be utilized during the initial stages of migration whereas the more conventional modes of neuronal migration would govern the later cortical development (Miyata and Ogawa, 2007). Interestingly, in cortical progenitor cells nestin is expressed in radially elongating G1 phase cells to become downregulated at the G2-M phase (Sunabori *et al*., 2008). Whether this is associated with the twisting of processes remains uncertain. Considering a report showing that the depletion of nestin does not hinder isolated kidney mesangial cell to migrate (Daniel *et al*., 2008), the role of nestin in cell migration seems highly context-dependent, similarly to the role of nestin in proliferation. Taken together, nestin seems influential for the cellular features typical for stem/progenitor cells, such as abilities to proliferate, migrate, and tolerate stress. Moreover, nestin may also play a role in defining the commitment of cells to differentiate. These findings place nestin at the crossroads of cell fate and indicate that nestin could function as a cellular signpost (Figure 8).

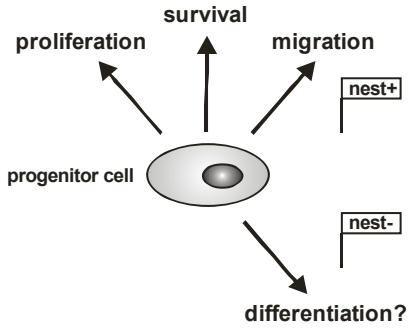

Figure 8. Nestin at the crossroads of cell fate. Nestin expression appears to coordinate the features and properties of progenitor cells.

4 Cyclin-dependent kinase 5 as a key determinant in development and disease

Cyclin-dependent kinase 5 (Cdk5) was identified in 1992 by virtue of its close sequence homology to Cdk1 (Hellmich *et al*., 1992, Lew *et al*., 1992, Meyerson *et al*., 1992). However, it became soon apparent that unlike the other members of the Cdk family, Cdk5 is not activated by cyclins and exhibits functions not related to cell cycle regulation. During almost two decades of research Cdk5 has been described to regulate a variety of proteins by phosphorylating serines and threonines with proline immediately downstream. Cdk5 has been implicated both in development and disease affecting a plethora of cellular activities including neuronal migration, synaptic transmission as well as neuronal death and survival.

4.1 Regulation of Cdk5

4.1.1 The regulatory subunits of Cdk5

Consistently to the other Cdks, Cdk5 has no enzymatic activity prior to the association with a regulatory protein. Distinct protein activators p35 and p39, showing no significant homology to cyclins, have been described to associate with and regulate the activity of Cdk5 (Tsai *et al*., 1994, Tang *et al*., 1995). p35 and p39 exhibit stringent expression patterns being heavily concentrated on postmitotic neurons (Tsai *et al*., 1994, Tang *et al*., 1995). Contrary to the perinatally lethal Cdk5 KO mice, discussed in detail in further sections, KO mouse model for p35 was revealed viable and fertile although displaying Cdk5 KO-like defects in CNS and increased tendency towards seizures (Chae *et al*., 1997). This indicates that p39 can possibly compensate some functions of p35. p35/p39 double KO mice, phenotypically indistinguishable from Cdk5 KO mice, confirmed that p35 and p39 were the main physiological activators of Cdk5 in CNS (Ko *et al*., 2001). Intriguingly, evidence has been presented that in bovine brain Cdk5/p35 would reside in a macromolecular complex containing possible inhibitory proteins (Lee KY *et al*., 1996). This indicates that the regulation of Cdk5 activity may extend beyond the interaction between Cdk5 and the activator proteins. An interesting feature of p35 and p39 is the N-terminal myristoylation which promotes the association of the Cdk5/p35 and Cdk5/p39 complexes with the cell membrane (Patrick *et al*., 1999, Kusakawa *et al*., 2000). In fact, although Cdk5 activated by either p35 or p39 is also present and has substrates in the cytoplasm and nucleus, approximately one third of Cdk5 substrates is membrane proteins or proteins associated with the cell membrane (Asada *et al*., 2008).

4.1.2 Phosphorylation of the Cdk5/p35 complex

The kinase activity of mitotic Cdks is negatively modulated by phosphorylation at T14 and tyrosine 15 (Y15). However, the activity of Cdk5 seems to increase when Cdk5 becomes phosphorylated at Y15 by c-Abl (Zukerberg *et al*., 2000) or Fyn (Miyamoto *et al*., 2008). Phosphorylation of T160/161 activates the mitotic Cdks but phosphorylation of the equivalent site on Cdk5 (S159) was suggested to sterically hinder the association between Cdk5 and p35 (Tarricone *et al*., 2001). CKIs seem not to have a prominent effect on Cdk5 activity (Lee MH *et al*., 1996).

The activation of Cdk5 launches an autoregulatory feedback loop in which the Cdk5-mediated phosphorylation of p35 results in ubiquitylation and proteosomal degradation of the activator protein (Figure 9; Patrick *et al*., 1998). Intriguingly, the phosphorylation of this Cdk5-specific site, T138 on p35, has been depicted to protect p35 from calpain-mediated processing to a more stable activator protein p25 (Kamei *et al*., 2007, discussed in 4.3.1). This negative feedback signal, limiting Cdk5 activity, highlights the tight regulation of Cdk5. On the other hand, a report depicting the phosphorylation-dependent stabilization of p35 further underlines the precise regulation of Cdk5 activity. PKCδ belonging to the family of novel PKCs was observed to phosphorylate and stabilize p35 in cortical neurons and positively regulate the radial migration of postmitotic neurons in a p35-dependent manner (Zhao *et al*., 2009). Besides modulating the stability of p35 (Patrick *et al*., 1998), the Cdk5-mediated phosphorylation of p35 has been reported to induce the activation and dissociation of Cdk5/p35 from the cell membrane in perinatal brain (Sato *et al*., 2007).

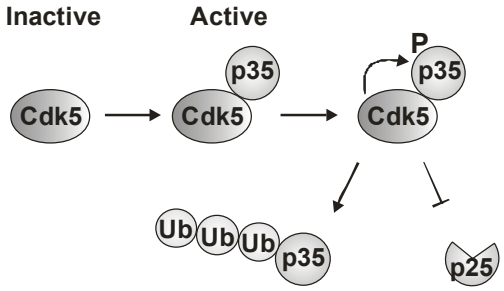

Figure 9. Autoregulatory feedback loop controlling Cdk5 activity. Cdk5-mediated phosphorylation of p35 on T138 reduces the stability of p35 by inducing p35 ubiquitylation and proteosomal degradation as well as suppresses the calpain-dependent cleavage of p35 to a more stable activator protein, p25, thus determining Cdk5 activity.

4.2 Established roles of Cdk5 in development and tissue homeostasis

4.2.1 Cdk5 is required for the development of central nervous system

The formation of cerebral cortex starts when the preplate, the first layer of postmitotic neuronal cells is split into a superficial marginal zone and a deeper subplate layer. Thereafter, the generation of cortex proceeds in an orchestrated fashion with inside-out layering of migrating cells when the newly born neurons migrate past the earlier cell layers towards the marginal zone. The expression of Cdk5 and its associated kinase activity have been described to substantially increase when the developing nerve cells are withdrawn from the cell cycle and be prominent in postmitotic migrating neurons (Tsai *et al*., 1993). With the help of Cdk5 KO mice, the essential role of Cdk5 in CNS development has started to emerge (Ohshima *et al*., 1996). The lack of Cdk5 leads to the disruption of neuronal layering in cortex and late embryonic or perinatal death. The inability of newborn cells to migrate through the layers of earlier generated neurons leading to inverted laminar organization points to the essential function of Cdk5 in nerve cell migration during corticogenesis (Gilmore *et al*., 1998). Similarly, the cerebellar layers are disrupted upon Cdk5 depletion (Ohshima *et al*., 1999). Thereafter, Cdk5-dependent regulation has been presented to govern the motility of several neuronal cell types including interneurons (Rakić *et al*., 2009) and oligodendrocyte precursors (Miyamoto *et al*., 2008).

Consistently with the proposed role of Cdk5 in migrating neurons, many of its identified substrates are cytoskeletal or cytoskeleton-associated proteins essential for the

neuronal cytoarchitecture (Table 3). Cdk5 is depicted as an important regulator of MT and MF dynamics. Somal translocation is a mode of neuronal migration where the extension of the leading process is followed by nuclear translocation, nucleokinesis, and the retraction of the trailing process. The dynamic regulation of MFs is essential for correct neuronal migration as the extension of the leading process requires actin treadmilling. The accumulation of Cdk5/p35 to actin rich growth cones (Nikolic *et al*., 1996) and the association of p39 with actin cytoskeleton (Humbert *et al*., 2000) hinted at the possibility of Cdk5 affecting MF structure. Cdk5 has been implicated as an effector of small GTPases involved in MF dynamics as it regulates Pak1 activity in a Rac-dependent manner (Nikolic *et al*., 1998). Identification of the Cdk5-p27 pathway was among the first direct evidences of the Cdk5-dependent regulation of MF binding proteins and MF dynamics in migrating neurons (Kawauchi *et al*., 2006). Cdk5-mediated phosphorylation was described to regulate the stability of p27, thus enabling the p27-mediated inhibition of Rho-kinase and suppression in the phosphorylation of actin-binding protein cofilin. Non-phosphorylated cofilin is active and able to control the MF reorganization (Kawauchi *et al*., 2006). Cdk5-mediated phosphorylation reduces also the ability of scaffolding protein Neurabin-1 to associate with MFs (Causeret *et al*., 2007) and, thereby, to affect the dynamics of MF network.

Protein	Outcome	Reference
Pak-1	MF dynamics	Nikolic *et al*., 1998
p27	Cofilin-mediated MF reorganization	Kawauchi *et al*., 2006
Neurabin-1	MF dynamics	Causeret *et al*., 2007
Ndel1	Dynein-mediated nuclear translocation along MT tracks	Niethammer *et al*., 2000, Sasaki *et al*., 2000
FAK	Stabilization of MT fork	Xie *et al*., 2003
DCX	MT dynamics	Tanaka T *et al*., 2004
NF-H, NF-M	Transport of NFs to axons and formation of axonal NF structures	Shetty *et al*., 1993, Sun *et al*., 1996, Sihag *et al*., 2007, Perrot *et al*., 2008

Table 3. Summary of Cdk5-regulated proteins controlling neuronal cytoarchitecture. Cdk5 regulates the dynamics of all the three cytoskeletal networks in developing CNS by regulating a variety of proteins.

Nucleokinesis is greatly dependent on the MT network which in association with the centrosome guides the transport of the nucleus towards the leading process (reviewed by Marin *et al*., 2010). Among Cdk5 substrates is Ndel1, a protein found in complexes with dynein and Lis1 (Niethammer *et al*., 2000, Sasaki *et al*., 2000). This finding is of particular interest as dynein-mediated retrograde transport has been implicated in nuclear translocation (Marin *et al*., 2010). Considering that Ndel1 and Lis1 have the capacity to affect dynein localization and that the inhibition of Ndel1 phosphorylation causes aberrant neurite morphology together with Ndel1 redistribution, it was speculated that Cdk5 plays a role in regulating the association between Ndel1 and dynein and, thus the dynein-based nuclear transport (Niethammer *et al*., 2000, Sasaki *et al*., 2000). In

addition, several factors, critical for the polymerization and stability of MTs, are known Cdk5 targets. FAK has been depicted essential for the stabilization of MTs between the centrosome and the nucleus. Phosphorylation of FAK at S732 by Cdk5 is critical for the enrichment of FAK at MTs and the sustainability of the fork-like MT structure without altering FAK activity (Xie *et al*., 2003). Cells overexpressing FAK S732A mutant loose the synchrony in nuclear movements, indicating that the MT fork links the nucleus and the centrosome, thereby enabling nucleokinesis. Strikingly similar cortical malformations in MT-associated doublecortin (DCX) mutated mice and Cdk5 KO mice prompted researchers to investigate the possible functional link between Cdk5 and DCX. Indeed, the Cdk5/p35 complex and DCX were observed to colocalize in the regions of developing brain containing migrating neurons (Tanaka T *et al*., 2004). The Cdk5-mediated phosphorylation of DCX at S729 decreased the ability of DCX to bind and stabilize the MTs, thereby affecting MT dynamics (Tanaka T *et al*., 2004). The drastic effects of Cdk5 ablation on cell migration could also stem from the disturbed regulation of cell-cell adhesion as Cdk5 by phosphorylating β-catenin is able to disrupt the interaction between β-catenin and N-cadherin, hence inhibiting adhesion (Kwon *et al*., 2000).

The cytoskeletal effects of Cdk5 in neurons extend beyond neuronal migration. The phosphorylation of NFs, IFs present in postmitotic neurons, is prominent in axons that have reached their targets and are forming synapses (reviewed by Sihag *et al*., 2007, Perrot *et al*., 2008). Phosphorylation is believed to modulate the transport of NFs from the cell soma to axons by regulating the interaction of NFs to MT motors. In addition, the formation of cross-bridges between NFs and other axonal structures, expanding the axonal caliber and conduction velocity, is affected by NF phosphorylation. NF-H and NF-M containing numerous KSP-repetitions in their C-terminal tails are heavily targeted by Cdk5 (Shetty *et al*., 1993, Sun *et al*., 1996). Therefore, it is presumable that Cdk5 could govern at least some aspects of NF modulation in neurons, although also other kinases, such as MAPKs, have been presented to phosphorylate NFs. However, the accumulation of phosphorylated NFs to cell bodies was observed to occur also in the cortical neurons of Cdk5-deficient mice (Ohshima *et al*., 1996). This was suggested to reside from a kinase cross-talk where the loss of Cdk5 activity would affect the functionality of other kinases including MAPKs (Kesavapany *et al*., 2004).

Despite that Cdk5 was initially shown not to promote the cell cycle progression, today it seems that Cdk5 has a general role in inhibiting the cell cycle in postmitotic neurons as well as in other cell types. The prominent nuclear localization of Cdk5 in cycling neurons is lost upon the cell cycle reentry triggered by stress or neurodegeneration (Zhang J *et al*., 2008). Recently the mechanism of Cdk5 to prevent the cell cycle progression was revealed not to stem from the kinetic functions of Cdk5 but rather from the ability of Cdk5 to sequester the cell cycle regulatory protein E2F1 (Zhang *et al*., 2010). The Cdk5/p35 complex was shown to hinder the association between E2F1 and cofactor DP1, thereby blocking the expression of cell cycle genes. The interaction between Cdk5/p35 and E2F1 relied solely on p35 with p25 and p39 showing no effect (Zhang *et al*., 2010).

Cdk5 has also been examined in adult neurogenesis. Although neurogenesis in adult brain is rare, it has been demonstrated to occur at the subventricular zone of lateral ventricles and at the subgranular zone of adult hippocampal dentate gyrus. In postnatal brain Cdk5 is essential for the migration of neuronal progenitors from the subventricular zone along the rostral migratory stream to the olfactory bulb, where they differentiate into neurons (Hirota *et al*., 2007). The cellular niches for neuronal progenitor cells in embryo

and adult organism are drastically different as in adult brain the newborn neuronal cells need to integrate into the preexisting system. Cdk5 has been demonstrated critical for the proper migration, neurite extension and synapse formation of hippocampal dentate granule cells in adult brain (Jessberger *et al*., 2008).

4.2.2 Synaptic functions of Cdk5 in central nervous system

Synaptic transmission enables the communication between neurons and the functionality of an organism. The connectivity is established by the reorganization of cytoskeletal elements leading to the formation of dendritic spines. Synaptic plasticity means that the efficacy of transmission is affected by the experience and is, therefore, thought to underlie the memory formation. Cytoskeletal dynamics enabling the changes in dendritic spine shape are essential for synaptic plasticity. Cdk5 has been observed to be enriched in synapses and phosphorylate synaptic proteins at both presynaptic and postsynaptic compartments, hence possessing widespread synaptic functions (reviewed by Angelo *et al*., 2006).

Cdk5 has been indicated to be involved in neurotransmitter release and recycling. For example, through the phosphorylation of voltage-dependent calcium channels, Cdk5 disturbs the formation of vesicle-associated protein complex at the channels, thereby impairing the Ca^{2+}-triggered exocytosis of synaptic vesicles and neurotransmitter release (Tomizawa *et al*., 2002). Cdk5 targets also proteins related to clathrin-dependent endocytosis including dynamin I and amphiphysin I (Tan *et al*., 2003, Tomizawa *et al*., 2003), through which it can affect the recycling of synaptic vesicles. In addition to these examples, a variety of Cdk5 substrates involved both in exo- and endocytosis has been characterized (Angelo *et al*., 2006). However, according to the results gathered from distinct CNS compartments, Cdk5 seems to regulate these processes both negatively and positively. Thus, thorough understanding of the functions of Cdk5 in vesicle dynamics requires further investigations. An important aspect of efficient synaptic transmission is the proper clustering of neurotransmitter receptors at the postsynaptic membrane. Cdk5-mediated phosphorylation has been described to regulate the clustering of a scaffold protein, postsynaptic density-95 (PSD-95, Morabito *et al*., 2004). Since PSD-95 is coupled to N-methyl-D-aspartic acid (NMDA) receptors, this could represent one mechanism of Cdk5 to modulate synaptic plasticity. In fact, the Cdk5-mediated phosphorylation of PSD-95 inhibits the binding of Src to PSD-95, thereby hampering the Src-dependent phosphorylation and endocytosis of NMDA receptors (Zhang S *et al*., 2008). Furthermore, structural remodeling of dendritic spines is relevant for synaptic plasticity. Cdk5 has been proposed to be influential for the modifications occurring in spine structure due to its ability to modulate the MF network occupying dendritic spines (Cheung *et al*., 2006, Lai and Ip, 2009). In addition to the surveys on molecular level, several *in vivo* studies have pointed to the role of Cdk5 in synaptic modulation and cognitive functions such as learning and memory (Cheung *et al*., 2006).

4.2.3 Cdk5 in skeletal muscle

Although Cdk5 activity is most prominent in postmitotic neurons, *Cdk5* and *p35* transcripts are also present in developing skeletal muscle (Philpott *et al*., 1997). During the differentiation of the C2 myoblast cell line Cdk5 exhibits dynamic changes in terms of protein amount and kinase activity both peaking when myoblasts fuse to multinucleated myotubes (Lazaro *et al*., 1997). The importance of Cdk5 in the progress of myogenesis was indicated by the enhanced differentiation following the ectopic expression of Cdk5

whereas the dominant negative form of Cdk5 (dnCdk5) significantly hampered the process. However, the increased Cdk5 activity alone was not able to shift the proliferative myoblasts to differentiation phase which means that additional myogenesis promoting cues are required (Lazaro *et al*., 1997). The results obtained with C2 myoblasts are consistent with the observations indicating that dnCdk5 disrupts muscle patterning and hampers the expression of myogenic regulatory proteins in *Xenopus laevis* embryos (Philpott *et al*., 1997). Furthermore, the expression of both Cdk5 and p35 increases in muscle as a consequence of nerve denervation (Fu *et al*., 2002) and necrotizing muscle injury (Sahlgren *et al*., 2003) implying that muscle regeneration, resembling embryonic myogenesis, may equally benefit from Cdk5 activity. Unfortunately, despite these interesting discoveries, the development, regeneration, and morphology of muscle tissue in Cdk5 KO mice has never been thoroughly addressed.

Very little is known about the myogenic targets of Cdk5. In our earlier studies we have observed that the expression pattern of nestin corresponds to the expression and activation of Cdk5 during myogenic differentiation and regeneration (Sahlgren *et al*., 2003). Indeed, we have identified nestin to be a specific Cdk5 substrate (Sahlgren *et al*., 2003). During the fusion of C2C12 myoblasts to multinucleated myotubes Cdk5 phosphorylates nestin at T316, thereby increasing the amount of unpolymerized nestin and enabling the reorganization of nestin filaments into parallel fibers. Most importantly, we have shown that nestin forms a scaffold for Cdk5/p35 where the activation of Cdk5 regulates the release of the Cdk5/p35 complex from nestin filaments (Sahlgren *et al*., 2003). The latter conclusion was based on the increased interaction between nestin and p35 in differentiating myoblasts upon Cdk5 inhibition. Cdk-like kinase Pctaire1 is another muscle-specific Cdk5 substrate (Cheng *et al*., 2002). Pctaire1 specifically interacts with p35 and is activated by Cdk5 but the exact functions of Pctaire1 as well as the Cdk5-dependent regulation remain obscure. In addition to nestin reorganization, myoblast elongation and fusion are characterized by the remodeling of MFs (O'Connor et al., 2008, Nowak *et al*., 2009) and MTs (Straube and Merdes, 2007). Although it is tempting to speculate, it is currently not known if Cdk5 serves to regulate these cytoskeletal modifications similarly than in migrating neurons. Besides the cytoplasmic effects of Cdk5, the rapid nuclear compartmentalization of Cdk5 detected shortly after the induction of differentiation is alluding to nuclear substrates (Lazaro *et al*., 1997). Whether Cdk5 regulates the cell cycle in postmitotic myoblasts remains to be explored.

Specific accumulation of Cdk5 is characteristic for NMJs (Sahlgren *et al*., 2003). It has been demonstrated to regulate the expression of acetylcholine receptors (AChRs) which ensure the synaptic transmission at the NMJs (Figure 10; Fu *et al*., 2001). A trophic factor neuregulin (NRG) is essential for the induction of proper synaptic gene expression. It functions through ErbB receptors to activate the downstream ERK signaling pathway leading to the transcription of AChR subunits at the NMJs. Cdk5/p35 complexes with ErbB receptors and phosphorylates ErbB3 in response to NRG (Fu *et al*., 2001). The inhibition of Cdk5 activity attenuated ErbB activation, induction of the downstream cascade and the expression of AChRs (Fu *et al*., 2001). Consistently, the ErbB2 and ErbB3 phosphorylation and ErbB activity were reduced in embryonic Cdk5 KO muscles although no similar reduction of AChR transcription was observed *in vivo,* probably due to redundant signaling mechanisms (Fu *et al*., 2005). However, the ablation of Cdk5 led to a disturbed clustering of AChRs, loss of Schwann cells and increased neurotransmission, depicting the versatile functions of Cdk5 in directing the formation and functionality of NMJs (Fu *et al*., 2005). The generation of the postsynaptic apparatus is initiated by means of muscle-intrinsic mechanisms before the muscle

becomes innervated (Lin *et al*., 2005). The nerve-derived agrin and acetylcholine (ACh) are utilized either to stabilize the postsynaptic apparatus or disperse the AChR clusters that have not been innervated respectively. Cdk5 has been presented to be activated by ACh agonists and to be required for the dispersion of the AChR clusters, hence explaining the larger AChR clusters detected in Cdk5 KO muscle (Lin *et al*., 2005).

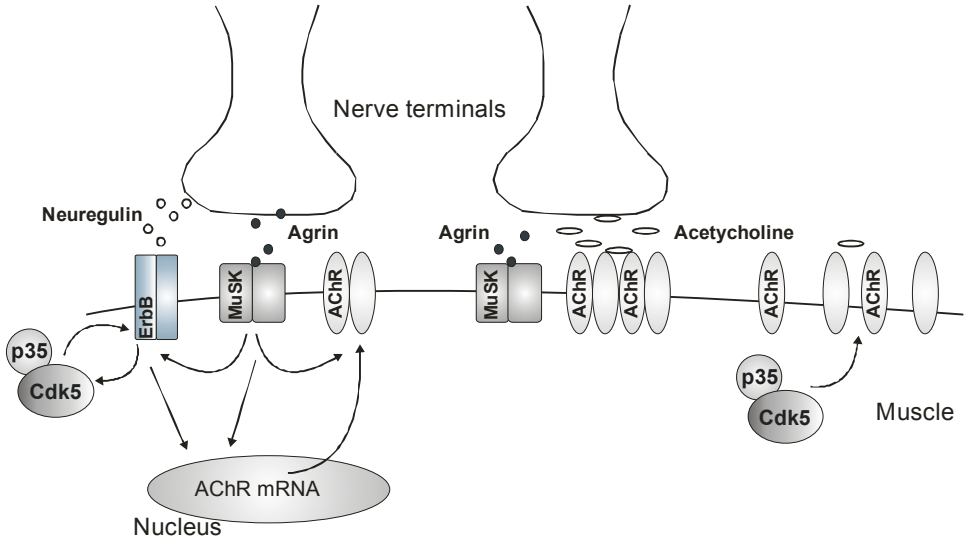

Figure 10. Cdk5 at the NMJs. Activation of Muscle-specific kinase (MuSK) receptor complex by agrin induces the clustering of AChRs at the NMJs. MuSK together with NRG and ErbB receptors triggers the expression or NMJ components, including AChR, in the nuclei located directly beneath the NMJs (Schaeffer *et al*., 2001). NRG induces Cdk5 activation, which is necessary for ErbB activation, thereby constituting a positive feedback loop where an increase in Cdk5 activity further activates ErbB receptors (Lee and Tsai, 2001). ACh and agrin provided by motor nerves serve as a negative and positive signal for NMJs respectively. It is proposed that agrin is not diffuse enough to overcome the dispersion effect of ACh in receptor clusters that are not apposed by a nerve terminal (Lin *et al*., 2005). Dispersion of the clusters far from nerve terminals is Cdk5-dependent.

4.2.4 Cdk5 is expressed in a multitude of cell types

Cdk5 was originally identified in CNS but has thereafter been observed in diverse cell types including Sertoli cells, lens cells, kidney podocytes and pancreatic β-cells, although the expression is described moderate compared to CNS (Rosales and Lee, 2006, Bertelli *et al*., 2007). Nevertheless, the importance of Cdk5 for the functionality of these cell types is obvious. Cdk5 has been implicated in glucose metabolism as it regulates the insulin secretion in pancreatic β-cells (Wei *et al*., 2005b) and glucose uptake in adipocytes (Lalioti *et al*., 2009). Expression of Cdk5 is prominent also in various types of cancer (Rosales and Lee, 2006). Considering the similarities between the formation of metastasis and cortical development in terms of cell migration, the influence of Cdk5 on the motile properties of prostate cancer cells was challenged. Cdk5 was delineated as governing the migration of prostate cancer cells in both cell culture and xenograft model systems (Strock *et al*., 2006). Apparent defects in MT organization were observed upon Cdk5 inhibition (Strock *et al*., 2006). Another survey with

glioblastoma cells presented Cdk5 as a positive upstream regulator of Akt and cell invasion stemming from the ability of Cdk5 to promote the GTPase activity of PI3K enhancer, a regulator of Akt pathway (Liu *et al*., 2008). The potent effect of Cdk5 on cell motility has been examined in a variety of cell models. Using Chinese hamster ovary cells Huang C and coworkers (2009) revealed the involvement of Cdk5 in talin stability and focal adhesion assembly. The Cdk5-mediated phosphorylation of talin head domain inhibited the binding of E3 ubiquitin ligase Smurf1 to talin, hence protecting talin from degradation. As the talin head domain, generated through calpain-mediated cleavage, has an essential role in integrin activation, Cdk5 provides a mechanism to stabilize focal adhesions and to set an optimal rate for cell migration (Huang C *et al*., 2009).

4.3 Deregulation of Cdk5

In addition to the pivotal role of Cdk5 in development, several lines of evidence indicate that aberrant Cdk5 activity contributes to neurodegenerative disorders. The calpain-mediated generation of p25 from p35 has been revealed to be responsible for the deleterious functions of Cdk5. The disease-associated formation of the Cdk5/p25 signaling complex reflects the requirements for tight regulation of Cdk5 activity.

4.3.1 Characteristics of p25

p25 is a proteolytic cleavage product of p35 generated by activated calpains (Kusakawa *et al*., 2000). Contrary to the short-lived p35 with a half-life less than 20 minutes, p25 is not readily degraded and has approximately two or three times longer half-life (Patrick *et al*., 1998, 1999). Moreover, this C-terminal cleavage product of p35 lacks the myristoylation signal located in the N-terminus of p35 (Patrick *et al*., 1999, Kusakawa *et al*., 2000) which increases the accumulation of the Cdk5/p25 complex into the cytoplasm and nucleus. Hence, it is no surprise that the prolonged activity of Cdk5 associated with the deregulation of subcellular localization has detrimental effects to cells. The active role of Cdk5/p25 in promoting cell death was published in two papers showing an increased amount of p25 in dying neurons (Kusakawa *et al*., 2000) and the induction of apoptosis in neuronal cultures following the overexpression of Cdk5 and p25 (Patrick *et al*., 1999). Dephosphorylation of T138, a Cdk5-specific phosphorylation site on p35, renders p35 more susceptible to calpain-mediated cleavage (Figure 8; Kamei *et al*., 2007). This particular site is phosphorylated in prenatal but not in adult brains and might function as a critical regulatory mechanism preventing the generation of p25 during development. Interestingly, the mutation of T138 to alanine stabilizes the short-lived p35 (Patrick *et al*., 1998) which could indicate that the rapid degradation of phosphorylated p35 would explain the inability of calpains to target it (Kamei *et al*., 2007). p29 is indicated as a cleavage product of the other Cdk5 activator protein, p39 (Patzke and Tsai, 2002b), but less research has been aimed at to characterize the functions of p29.

4.3.2 The Cdk5/p25 signaling complex in neurologic diseases

The pathological hallmarks of Alzheimer's disease (AD) are the accumulation of β-amyloid (Aβ) peptides to extracellular amyloid plaques and the generation of intracellular neurofibrillary tangles composed of hyperphosphorylated tau (reviewed by Cruz and Tsai, 2004). The resulting neuronal death and the loss of neurons incur the symptoms of AD, cognitive and motor dysfunction. Today the calpain-mediated cleavage of p35 to

p25 is known to play a significant role in the pathogenesis of AD. Cdk5/p25 is able to influence the processing of β-amyloid precursor protein (APP) to Aβ as Cdk5 transcriptionally regulates the APP cleaving enzyme (Wen *et al*., 2008) and the Cdk5-mediated phosphorylation of APP increases the secretion of Aβ (Cruz and Tsai, 2004). Furthermore, the Cdk5/p25 complex efficiently hyperphosphorylates microtubule-associated protein tau reducing the ability of tau to bind MTs, thus causing MT destabilization and aggregation of tau to neurofibrillary tangles (Patrick *et al*., 1999, Cruz *et al*., 2003). Combined with the Cdk5/p25-mediated pronounced phosphorylation and disassembly of the NF network (Patrick *et al*., 1999), Cdk5/p25 induces rather comprehensive cytoskeletal disruption in neurons. The generation of transgenic mice producing p25 has confirmed that the cytoskeletal disturbances stemming from the hyperactive Cdk5 are similar to neurodegenerative diseases, including AD, thus linking p25-induced enhanced Cdk5 activity even more solidly to disease pathologies (Ahlijanian *et al*., 2000). In addition to AD, the deregulation of Cdk5 has been implicated in other neurodegenerative disorders including Amyotrophic lateral sclerosis, Parkinson´s disease and ischemia (Patzke and Tsai, 2002a, Cruz and Tsai, 2004).

4.3.3 Influence of Cdk5/p25 on apoptotic responses

The conversion of p35 to p25 has been shown to be triggered by a variety of neurotoxic conditions including oxidative stress (Strocchi *et al*., 2003), exposure to Aβ peptides (Lee *et al*., 2000), glutamate-induced excitotoxicity (Tang *et al*., 2005), ischemia (Wang et al., 2003) and ER stress (Saito *et al*., 2007). Neurotoxic insults often trigger neuronal apoptosis in many neurological disorders. Therefore, in defining the etiology of neurodegenerative diseases, efforts have been devoted to understand the Cdk5/p25-induced cellular perturbations leading to apoptotic cell death. During the past years Cdk5 activity has been shown to modulate the functionality of several proteins during neurotoxicity-induced apoptosis (Figure 11). For example, the regulation of survival-promoting molecular machinery can have drastic effects on cell fate. The phosphorylation of transcription factor MEF2 by Cdk5/p25 induces the caspase-dependent MEF2 degradation, hence inhibiting the transcriptional activity and the ability of MEF2 to protect cells from apoptosis (Gong *et al*., 2003, Tang *et al*., 2005).

Neuronal stress often coincides with the reactivation of cell cycle regulators. However, the cell cycle reentry of postmitotic neurons under cell stress leads to cell death with no signs of cell division. In addition, DNA damage has been associated with various neurodegenerative conditions. Although the mechanisms, how the cell cycle reentry or DNA damage affect the progress of neuronal cell death, are still somewhat unclear, these processes seem to have an active role in neuronal apoptosis (reviewed by Kim and Tsai, 2009). Cdk5 has been implicated to participate in the regulation of both apoptotic cell cycle activity and DNA damage. The phosphorylation of Rb by the Cdk5/p25 signaling complex accompanied with the upregulation of E2F-responsive genes is among the earliest events of neuronal cell death (Hamdane *et al*., 2005).

The aberrant induction of p25 has been described to result in double-strand DNA breaks (Kim *et al*., 2008). p25 was found to interact with the catalytic domain of HDAC1 and inhibit the enzymatic activity of HDAC1 although the detailed mechanism remains elusive (Kim *et al*., 2008). The acetylation decreases the ability of histones to bind to DNA leading to hampered chromatin compaction, increased susceptibility to DNA breaks and deregulated gene expression. Apurinic/apyrimidinic endonuclease 1 (Ape1) is

essential for the repair of DNA damaged by oxidative stress or genotoxic compounds. Cdk5 was recently illustrated to phosphorylate Ape1 on T232, thereby reducing Ape1's endonuclease activity and promoting the accumulation of DNA damage leading to neuronal death (Huang *et al*., 2010). Remarkably, the Cdk5/p25-mediated phosphorylation of Ape1 was detected to be more deregulated as it was not affected by the interaction between the kinase complex and Ape1 similarly than the Cdk5/p35-mediated phosphorylation of Ape1 (Huang *et al*., 2010). The PI3K-like kinase ATM (ataxia telangiectasia mutated) coordinates the downstream DNA-damage responses by activating the p53 tumor suppressor protein which induces the expression of death related target genes such as *puma* and *bax*. The treatment of neuronal cells with DNA-damaging agents induces rapid induction in Cdk5 activity and the Cdk5-mediated phosphorylation of ATM at S749 which is required for ATM activation (Tian *et al*., 2009). The attenuation of this pathway prevents the transcription of proapoptotic p53 target proteins and protects neurons from cell death (Tian *et al*., 2009). p53 is reported also to be a direct Cdk5 substrate. In response to genotoxic or oxidative stress the Cdk5-mediated phosphorylation disrupts the interaction between p53 and Hdm2, an E3 ubiquitin ligase of p53, thereby stabilizing p53 (Lee *et al*., 2007). Moreover, the Cdk5-mediated phosphorylation promotes the acetylation and transcriptional activity of p53, leading to the increased expression of proapoptotic p53 target genes stimulating the intrinsic mitochondrial pathway of apoptosis (Lee *et al*., 2007).

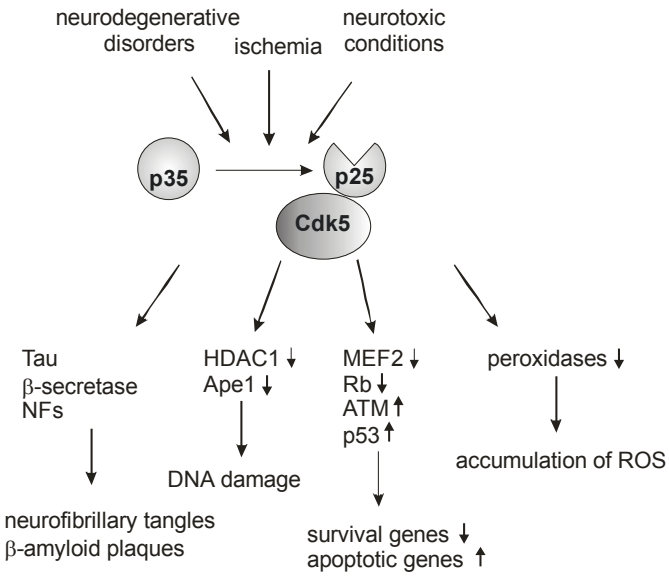

Figure 11. Cdk5/p25-mediated neurodegeneration. Generation of p25 is linked to neurodegenerative disorders and can be triggered by a variety of neurotoxic insults. The Cdk5/p25 complex induces severe perturbations in the functionality of several proteins, thereby evoking apoptotic cell death (modified from Ikiz and Przedborski, 2008).

Oxidative stress resulting in mitochondrial dysfunction contributes to the early phases of AD. Similarly, the treatment of neuronal cells with glutamate or Aβ leads to the accumulation of ROS and mitochondrial depolarization. Cdk5 has been uncovered to inhibit the activities of peroxidases, peroxiredoxin I and II, through phosphorylation (Sun

KH *et al*., 2008). Thus, the attenuation of Cdk5 activity reduces the amount of accumulating ROS and prevents the mitochondrial damage. As the mitochondrial depolarization leads to an increase in the intracellular Ca^{2+} levels, the authors suggested that this would further strengthen the initial death-promoting Cdk5/p25 signal (Sun KH *et al*., 2008). To sum up, the Cdk5/p25 complex seems to have prominent proapoptotic effect residing from aberrant regulation of a variety of proteins. However, it remains to be explored whether Cdk5/p25 promotes distinct cell death cascades depending on the type of stress or the type of tissue where the hyperactivation occurs.

Contrary to the seemingly neurotoxic functions of Cdk5/p25 summarized in figure 11, Cdk5 has also been implicated as a regulator of neuronal survival. For example, although a transient activation of ERK1/2 is beneficial for proliferation and differentiation, sustained ERK1/2 activation seems to have detrimental effect in neurons. The Cdk5-dependent inhibition of MEK1, an upstream activator of ERK1/2, promotes cell survival in cortical neurons (Zheng *et al*., 2007). Moreover, the Cdk5/p35-mediated phosphorylation suppresses the activity of proapoptotic JNK3 (Li *et al*., 2002) whereas the Cdk5-mediated phosphorylation of Bcl-2 promotes the antiapoptotic functions of Bcl-2 in neurons (Cheung *et al*., 2008). Antiapoptotic functions of the Cdk5/p35 complex extend also to non-neuronal cells as Cdk5/p35 has been described to protect podocytes from cell death (Brinkkoetter *et al*., 2010). These results underline the critical role of Cdk5 in life and death decisions emphasizing that moderate Cdk5 activity may enhance survival during developmental processes. The cleavage of p35 to p25 resulting in a prolonged activation together with an aberrant subcellular distribution and nuclear accumulation of Cdk5 could define a mechanism through which the activity of Cdk5 turns pathological (O'Hare *et al*., 2005). This accentuates the need for a strict regulatory machinery for Cdk5 to prevent unwarranted and harmful activity.

4.3.4 Surprising cognitive benefits of the Cdk5/p25 complex

p25 overexpressing mouse models have been widely utilized in surveying the pathogenesis of AD. Intriguingly, the transient expression of p25 in mice was discovered to have unexpected effects as it did not cause neurodegeneration as was the situation with the prolonged overexpression of p25, but instead improved learning and memory (Fischer *et al*., 2005). These effects correlated with increased hippocampal long term potentiation (LTP), NMDA receptor signaling, dendritic spine density and the number of synapses. In contrast, the prolonged production of p25 led to a reduction in the number of synapses and impaired the formation of new memories (Fischer *et al*., 2005). These observations led to a hypothesis that limited amount of p25 would be produced during aging to compensate for the loss of Cdk5 activity and facilitate cognitive functions, hence ameliorating the early memory defects. However, the exposure to AD risk factors would increase the amount of p25 over the critical threshold and invert eventually the situation detrimental (Fischer *et al*., 2005). Similar results was achieved with two mouse strains overexpressing low levels of p25, although a prominent sexual dimorphism with only females showing improved learning was detected (Ris et al., 2005). These interesting results suggest that the calpain-mediated generation of p25 does not automatically lead to neuronal death but the tightly controlled generation of p25 can actually be beneficial under certain circumstances.

5 An overview of the regulation and functions of protein kinase C ξ

PKCξ was originally observed to be related to, but distinct from, the other members of the PKC family (Ono *et al*., 1989). Hence, it was classified into the atypical subfamily of PKCs (aPKCs) together with PKCλ/ι (PKCλ is the mouse homolog of human PKCι). The major difference between the PKC subfamilies, conventional (cPKC), novel (nPKC) and atypical PKCs, is the mechanism of activation (reviewed by Hirai and Chida, 2003, Moscat *et al*., 2009). Contrary to the cPKCs and nPKCs, the activation of aPKCs does not require Ca^{2+} or diacylglycerol, but is still dependent on the release of the pseudosubstrate (PS) sequence from the substrate-binding cavity and the phosphorylation of the kinase domain. Lipid components, such as phosphatidylinositol (3,4,5)-trisphosphate, are presented to enable the release from PS-induced autoinhibition whereas 3-phosphoinositide-dependent protein kinase 1 (PDK1), a downstream effector of PI3K, is depicted to phosphorylate the kinase domain of PKCξ (Figure 12). The PB1 domain is a conserved protein interaction module present in the N-terminus of PKCξ and responsible for many of the PKCξ interactions. PKCξ has been implicated to complex with adaptor proteins, such as p62/sequestosome 1 and Par-proteins modulating PKCξ activity and the outcome of PKCξ-dependent signal transduction (reviewed by Etienne-Manneville and Hall, 2003a, Hirai and Chida, 2003, Moscat *et al*., 2009).

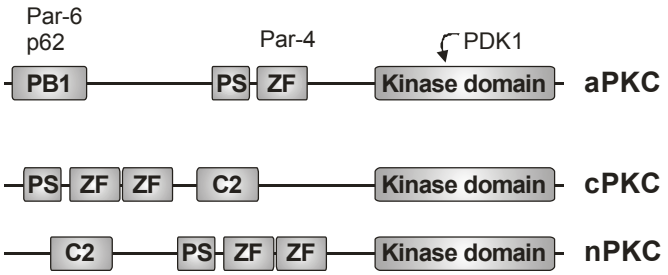

Figure 12. Regulatory domains of aPKCs. Through the N-terminal protein interaction domain, PB1, as well as the zinc finger domain (ZF) PKCξ interacts with adaptor proteins. PDK1 targets the C-terminal catalytic domain whereas lipid components mediate the release from PS-dependent autoinhibition. In contrast to cPKCs and nPKCs. aPKCs lack the Ca^{2+}-binding C2 domain and have only one ZF domain. These structural differences may explain the divergent mechanisms of PKC activation (modified from Moscat *et al*., 2009).

The involvement of PKCξ has been established important for a variety of signaling cascades, for example MAPK pathway (Diaz-Meco and Moscat, 2001, Cohen *et al*., 2006). It has also been found to function in insulin signaling and glucose uptake. Through phosphorylation PKCξ modulates the ability of insulin receptor substrates to transmit the signal from insulin receptors (Ravichandran *et al*., 2001, Moeschel *et al*., 2004, Lee *et al*., 2008) whereas through MF remodeling PKCξ regulates the translocation of glucose transporter 4 containing vesicles to the cell membrane (Liu *et al*., 2006). In addition, PKCξ is depicted to promote the activity of p70 ribosomal S6 kinase (p70S6K) which in response to mitogenic stimuli controls the translational machinery (Romanelli *et al*., 1999). Furthermore, PKCξ participates in the regulation of

NFκB activation. It has been reported to enable the interaction between NFκB complex and transcriptional coactivator CBP by phosphorylating the RelA subunit in NFκB complex as well as to enhance the activation of IKK through phosphorylation (Lallena *et al*., 1999, Duran *et al*., 2003).

Interestingly, PKCξ has been characterized to act as a mediator of diverse developmental processes. It affects the nerve growth factor-induced differentiation of PC12 pheochromocytoma cells (Wooten *et al*., 1994, 1999). Overexpression of PKCξ enforces an erythroid phenotype in leukemic cells suggesting that PKCξ might promote also erythropoiesis (Mansat-De Mas *et al*., 2002). Moreover, PKCξ expression has been shown to correlate with the morphological alterations in differentiating melanocytes (Kim JH *et al*., 2010) and in neonatal myocardial tissue (Centurione *et al*., 2003). In the former model PKCξ was documented to regulate neurite extension whereas the latter effect was suggested to result from PKCξ-dependent modulation of cell growth and apoptosis. PKCξ KO mice have provided additional insight into the developmental functions of PKCξ as these mice display a delay in the development of secondary lymphoid organs: lymph nodes, Peyer's patches and spleen (Martin *et al*., 2002). PKCξ is also important for B-cell proliferation and survival and the ablation of PKCξ results in reduced B-cell numbers in secondary lymphoid organs (Martin *et al*., 2002). In addition, the differentiation of PKCξ-deficient Th2 cells is compromised further emphasizing the developmental functions of PKCξ (Martin *et al*., 2005).

aPKCs have been presented to critically regulate cell polarization, an essential cellular event underlying various developmental processes. The complexes between aPKCs and Par-proteins have been implicated in the regulation of MT organization, thus assisting the establishment of cell polarity, important for the asymmetric cell division and cell migration (Etienne-Manneville and Hall, 2003a). For example, the chemotaxis of macrophages is critically regulated by PKCξ (Guo *et al*., 2009) and the migration of astrocytes is affected by the PKCξ/Par-6 complex determining the reorientation of centrosome towards the direction of cell elongation (Etienne-Manneville and Hall, 2001). The latter was later reported to occur as a result of the PKCξ-mediated phosphorylation of Glycogen synthase kinase 3β (GSK-3β) leading to the inhibition of GSK-3β activity (Etienne-Manneville and Hall, 2003b). This reduced the ability of GSK-3β target protein adenomatous polyposis coli to interact with the plus ends of MTs, hence affecting MT organization. Consistently in migrating cerebellar granule neurons the PKCξ/Par-6 complex localizes to the centrosome and regulates the dynamics of MT network, thereby enabling the coordinated movement of the centrosome prior to the somal translocation of the nucleus (Solecki *et al*., 2004). An accumulation of Aurora A around the centrosome in elongating neurites has been depicted to result from PKCξ-mediated phosphorylation of Aurora A (Mori *et al*., 2009). The activated Aurora A phosphorylates Ndel1 and facilitates MT extension into neurite processes. These results place PKCζ in a central role in the regulation of cell migration.

OUTLINE OF THE STUDY

The concept of IFs as regulatory scaffolds for signaling determinants had been recently introduced when this PhD thesis work was conducted in autumn of 2003. Previous work in our laboratory, focused on determining the phosphorylation-mediated regulation of the IF protein nestin, had revealed besides Cdk5 as a specific nestin kinase but also a strong interaction between nestin and the Cdk5/p35 signaling complex. Therefore, the emphasis of my PhD studies was to investigate whether nestin functions as a scaffold for the Cdk5/p35 complex affecting the cellular events involving Cdk5 activity. We sought to define how nestin affects the key features of Cdk5 and p35, including the kinase activity, the subcellular localization, and the proteolytic processing of p35. In addition, by addressing the potential role of nestin as a regulator of the Cdk5/p35 signaling complex, we pursued to obtain novel information about the cellular functions of nestin of which very little was known at that time. Furthermore, the importance of phosphorylation in the regulation of IF organization and turnover, prompted us to investigate whether Cdk5 regulates the stability and turnover of its own scaffold. To answer these challenges we decided to utilize two different model systems characterized by the nestin expression and Cdk5 activity, the hydrogen peroxide (H_2O_2)-triggered oxidative stress in neuronal progenitor cells and the differentiation of myoblasts to multinucleated myotubes.

The mechanisms regulating Cdk5 activity have been extensively studied in neuronal context. However, despite the specific activation of Cdk5 in the developing and regenerating muscle as well as at the NMJs, the regulation of Cdk5 in myogenic tissue has never been thoroughly addressed. We endeavored to shed light on the upstream regulatory cascades directing the activation of Cdk5 in differentiating myoblasts.

The aims of this thesis were:

- To investigate the dynamic crosstalk between nestin and the Cdk5/p35 signaling complex in model systems characterized by Cdk5 activity; neuronal stress and myogenic differentiation.
- To identify the regulatory mechanism controlling the activation of Cdk5 in differentiating myoblasts.

EXPERIMENTAL PROCEDURES

1 Cell culture

1.1 Maintenance of cell lines (I-III)

All the cell lines used were maintained in a humidified 5% CO_2 atmosphere at 37ºC. ST15A neuronal progenitor cell line is isolated from the neonatal rat cerebellum (Frederiksen *et al*., 1988). These cells can be induced to differentiate towards neuronal or glial cell type but in our experiments ST15A cells were grown under conditions keeping them in proliferative progenitor cell-like state. ST15A cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS), 2 mM L-Glutamine, and antibiotics (penicillin and streptomycin).

C2C12 cell line is a subclone from a mouse myoblast cell line established from the thigh muscle of adult mouse (Yaffe and Saxel, 1977). This cell line was maintained in DMEM supplemented with 10% FBS, 2 mM L-Glutamine, and antibiotics (growth medium). C2C12 myoblasts provide an excellent model system to investigate myogenesis as these cells can be induced to differentiate and fuse to multinucleated myotubes expressing characteristic muscle proteins. Differentiation of C2C12 myoblasts was induced by mitogen removal by switching subconfluent cell cultures to DMEM supplemented with 1% FBS, 2 mM L-Glutamine, and antibiotics (differentiation medium).

HeLa human epithelial cervical cancer cells, Cos7 monkey kidney cells, HEK293 human embryonic kidney cells and SW13 human adenocarcinoma cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-Glutamine, and antibiotics. SW13 cells expressing vimentin in a mosaic pattern were subcloned by dilution cloning. Cell clones were upscaled and examined for vimentin expression by Western blotting. Vimentin-negative and vimentin-positive subclones were selected for experimentation.

1.2 Isolation and culture of primary myoblasts (II, III)

Cultures of primary myoblasts were established from the limb skeletal muscles of 2-day-old wild type and vimentin KO 129/SV mice (Colucci-Guyon *et al*., 1994). For nestin downregulation studies primary myoblasts were isolated from the limb skeletal muscles of 2-day-old FVB-n mice. In both cases muscle tissue was minced and enzymatically digested by incubation in 0.2% type XI collagenase (Roche Diagnostics) and 0.1% trypsin at 37°C for 45 min. The resulting slurry was filtered to remove large pieces of tissue and rinsed with growth medium (Hams F-10 [Sigma] supplemented with 15% FBS, 2 mM L-glutamine, antibiotics, and 2.5 ng/ml fibroblast growth factor-β [Promega]). Cells were centrifuged at 1000 x *g* for 5 min, resuspended in growth medium, and plated into tissue culture dishes. After the attainment of 80% confluence, the differentiation was induced by replacing growth medium with differentiation medium (DMEM supplemented with 2% FBS, 2 mM L-glutamine, and antibiotics).

1.3 Experimental treatments (I-III)

Oxidative stress was induced in ST15A neuronal progenitor cells by 100 μM H_2O_2. 20 μM Z-VAD (Sigma) was applied to H_2O_2-treated cells 3 h before harvesting to inhibit caspases whereas 10 μM MG132 (Peptide institute) was applied 6 h before harvesting to inhibit proteasomes. In order to inhibit Cdk5 during oxidative stress, Roscovitine (Calbiochem) was added to a final concentration of 10 μM 3 h after the induction of stress (time points 5, 6 and 8 h). For analyzing the p35 turnover the proteasome inhibitor MG132 and the protein synthesis inhibitor cycloheximide (CHX; Sigma) were used at concentrations of 20 μM and 30 μg/ml respectively for indicated time periods.

To specifically inhibit the activity of PKCξ, C2C12 and mouse primary myoblasts were induced to differentiate in the presence of 15-20 μM PS peptide inhibitor (PS; Myr-SIYRRGARRWRKL; MilleGen Prologue Biotech). Scrambled peptide (Scr-P; Myr-RLYRKRIWRSAGR) was used as a control to rule out the unspecific effects of peptide treatment. Both of the peptides were linked to a myristoyl group to facilitate the transport through the cell membrane. Calphostin C was used to specifically inhibit the cPKCs and nPKCs. The PKC inhibitor chelerythrine (Calbiochem) was used at a concentration of 5 μM. For detecting the role of Cdk5 in myogenic differentiation C2C12 cells were treated with 10 μM Roscovitine from the induction of differentiation. The cleavage of p35 to p25 was analyzed by modifying the activity of calpains. Calpain activity was induced in C2C12, HeLa and in both vimentin-positive and negative SW13 cells by adding 10 μM calcium ionophore A23187 (Calbiochem). The inhibition of calpain activity in C2C12 cells was obtained with 15 μM calpain inhibitor III (Calbiochem). The proteasomal inhibitor MG132 was used at a concentration of 20 μM.

The effect of NMJ-related signaling on nestin protein levels in C2C12 myotubes (differentiated for 96 or 120 hours) was investigated by treating the myotubes with 10 ng/ml of recombinant rat C-terminal agrin (R&D Systems) for 16h followed by a treatment with 50 ng/ml of recombinant human NRG-β1 extracellular domain (R&D Systems) for indicated times. Prior to the addition of NRG myotubes were carefully rinsed. To inhibit Cdk5 activity 10-20 μM Roscovitine was added to myotubes 30 min before the addition of NRG. Lysates were harvested 12 hours after the NRG induction.

2 Plasmid constructs (I-III)

To downregulate nestin, siRNA vector was generated using the pSUPER vector (Oligoengine) and the specific nestin sequence, CTCTCCCTGACTCTACTCC, according to the manufacturer's protocol (Brummelkamp *et al*., 2002). The scrambled sequence that was used as a control did not correspond to any known gene in the databases and had the following sequence: GCGCGCTTTGTAGGATTCG. The series of GFP-tagged nestin deletion mutants (Figure 13) was prepared by Dr. Ying-Hao Chou (Northwestern University, Chicago, USA). These plasmids encoded the first 314 (nest-314), 640 (nest-640), 1177 (nest-1177) and 1893 (nest-1893, corresponding to the full length nestin) amino acids of the rat glioma nestin sequence. In addition, GFP-tagged nestin mutant representing the last 332 amino acid residues of the C-terminal tail domain was constructed using the hamster nestin sequence (nest-T332). All nestin cDNAs were cloned into Clonetech's pEGFP-C1 vector.

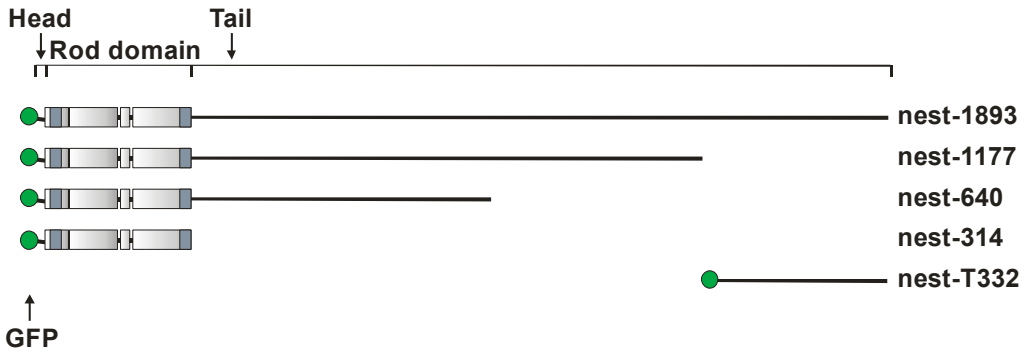

Figure 13. Schematic presentation of the nestin mutants.

p35 and both the WT Cdk5 and dnCdk5 cloned into pcDNA3.1 His vector expressing a His_6 tag and aXpress epitope were kindly provided by Dr. Harish Pant (National Institutes of Health, Bethesda, USA). HA-tagged ubiquitin was a kind gift from Dr. Dirk Bohmann (EMBL, Heidelberg, Germany). Myc-tagged p35 was purchased from Addgene. Point mutation to this vector was made using QuikChange site-directed mutagenesis kit (Stratagene) and confirmed by sequencing. The FLAG-tagged PKCξ was kindly provided by Dr. John Blenis (Harvard Medical School, Boston, USA).

3 Transient transfections (I-III)

ST15A, Cos7, HEK293, HeLa and SW13 cells were transiently transfected with electroporation. For this procedure cells were collected, resuspended in OptiMEM (Gibco) and placed in 0.4 cm electroporation cuvettes (BTX) where plasmid DNA had been added. Cells were subjected to a single electric pulse (220 V, 975 µF) using a Bio-Rad Gene Pulser electroporator followed by dilution in DMEM supplemented with 10% FBS, 2 mM L-Glutamine and antibiotics. Cells were incubated for 24-48 h prior to experimental procedures. For ST15A transfections the following plasmid constructs were used: GFP-tagged nest-314, nest-640, nest-1177, nest-1893, nest-T332, pSUPER nestin, pSUPER scrambled, HA-tagged ubiquitin, His-tagged p35 and both WT and dnCdk5. Empty pcDNA3.1 and pEGFP-C1 vectors were used as controls. Cos7 cells were transfected equally with electroporation with the exception that OptiMEM was supplemented with 5% FBS. FLAG-tagged PKCξ and His-tagged p35 were used in Cos7 transfections. HEK293 cells were transfected with constitutively active PKCξ together with either WT or S33A-mutated myc-tagged p35 in order to conduct *in vivo* ^{32}P labeling and the comparison of WT and mutant p35 phosphopeptide maps. HeLa cells and SW13 cell clones were transfected with plasmids encoding either GFP-tagged nest-640 or plain GFP together with WT Cdk5 and myc-tagged p35.

C2C12 and mouse primary myoblasts were transfected using different commercial transfection reagents. In order to downregulate PKCξ primary myoblasts were transfected with 20 or 80 pmol of PKCξ siRNA pool (Santa Cruz Biotechnology) using the Lipofectamine plus reagent (Life Technologies) according to the manufacturer's instructions. Scrambled siRNA was used as a control. 20 h after the transfection,

differentiation was induced by switching the cells to differentiation medium. The depletion of nestin was achieved in C2C12 and primary myoblasts using RNAi oligos purchased from Qiagen. Nestin RNAi and AllStars negative control RNAi were transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. After 8-9 h the medium was replaced with differentiation medium or growth medium when aiming for MTS assay. For plasmid transfections C2C12 cells were plated on 12-well plates and transfected with JetPei reagent (Polyplus-transfection) according to the manufacturer's instructions with a total amount of 2 µg DNA per well. Differentiation was induced 6-8 h following the transfection. Four double transfections C2C12 cells were transfected first with RNAi oligos for 6 h followed by a JetPEI-based transfection with dnCdk5 for additional 6 h.

4 Microscopic analyses (I-III)

For immunofluorescence analysis ST15A cells were transfected with nestin constructs together with Cdk5 and p35 and grown on coverslips. Following the treatment with H_2O_2, cells were washed with phosphate-buffered saline (PBS), fixed with 3% paraformaldehyde (PFA) for 10 min at room temperature (RT), permeabilized for 5 min in 0.5% Triton X-100 in PBS, and incubated for 1 h in blocking solution (1% bovine serum albumin [BSA] in PBS). Cells were labeled with an antibody against p35 and p25 (C-19, Santa Cruz Biotechnology). After washes with PBS cells were incubated with secondary antibody, Alexa 568 (Molecular Probes), for 1 h, washed and mounted with 80% glycerol. Images were collected using a Zeiss LSM 510 META laser scanning confocal microscope equipped with a Plan-Apochromat 63x/1.4 oil DIC objective. Images are maxprojections generated from 8-12 (0.8-1 µm thick) *z*-sections using Zeiss LSM 3.0 software. Phase contrast images of H_2O_2-treated ST15A cells fixed with 3% PFA were captured with Zeiss Axiovert 200M widefield microscope.

To investigate the morphological and biochemical features of differentiation C2C12 and mouse primary myoblasts differentiating on coverslips were fixed in 3% PFA for 10 min at RT and permeabilized with 0.1-0.5% Triton X-100 in PBS for 10 min at RT. Non-specific binding sites were blocked by incubation in 1-2% BSA in PBS for 30-60 min at RT. Cells were subsequently stained for 1-2 h with primary antibodies against myosin heavy chain (MHC), nestin and p35, after which the coverslips were rinsed three times with PBS or with PBS containing 0.5% Triton X-100 and stained for 40-60 min with secondary antibodies tagged with fluorescent dyes (Alexa 488 and Alexa 568, Molecular Probes). Cells were washed and mounted in Vectashield (Vector Laboratories). Samples were visualized using a Zeiss LSM 510 META laser scanning confocal microscope.

5 Viability and proliferation assays

5.1 DAPI labeling for detection of apoptotic nuclear morphology (I)

ST15A cells were transfected either with nestin constructs or with empty GFP vector and plated on coverslips. In order to distinguish the apoptotic cells in a population of H_2O_2-treated transfected ST15A cells, cells were fixed with 3% PFA, stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma) for 1 min, washed with PBS and mounted in 80% glycerol. The morphology of nuclei was visualized with Leica DMRE microscope

equipped with Hamamatsu Photonics ORCA CCD camera. Apoptotic nuclei were identified by the condensed chromatin and the disturbed nuclear morphology.

5.2 MTT assay for detection of apoptosis (I)

Cellular damage gradually leads to the inability of cells to provide energy for metabolic cell functions. Therefore, metabolic activity assays, often measuring mitochondrial activity, can be utilized in determining the degree of cell death. 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is widely used to quantitate cytotoxicity. Yellow MTT is reduced to purple formazan by mitochondrial enzymes i.e. by the cells that have functional mitochondria. Control and H_2O_2-treated transfected ST15A cells in 12-well plates were washed with Hepes-buffered saline solution and subsequently incubated for 15 min with MTT (0.5 mg/ml in Hepes-buffered saline solution) at 37°C. The MTT-solution was removed and the formazan product formed was solubilized in 5% sodium dodecyl sulfate (SDS) overnight at RT. The results were quantified spectrofotometrically.

5.3 Propidium iodide labeling of apoptotic nuclei (I)

The degree of apoptosis among ST15A cells incubated with 100 μM H_2O_2 for 6 h was determined by flow cytometry assay of propidium iodide (PI)-stained nuclei. PI is a DNA intercalator that can be used to determine the DNA content within the cells. The relative DNA content is reduced in apoptotic cells. For the analysis both control and H_2O_2-treated cells were harvested, washed with PBS and suspended in 300 µl of hypotonic solution containing PI (10 µg/ml PI in 0.1% sodium citrate and 0.1% Triton X-100). Cells were incubated in solution overnight at 4°C and analyzed by FACSCalibur flow cytometer (BD Pharmingen).

5.4 MTS assay for analysis of proliferation rate (III)

Proliferating cells are metabolically more active than non-proliferating cells. Thus, metabolic assays are also suitable for the detection of proliferation. Nestin was downregulated by RNAi and the proliferation of nestin-depleted C2C12 myoblasts was determined by MTS assay. Equal amounts of cells transfected either with nestin RNAi or with scrambled RNAi were seeded on 12-well plates 24 hours after the transfection and cultured in growth medium for additional 24 hours. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS; Promega) reagent supplemented with phenazine ethosulfate (PES; Sigma) was added to each well and cells were incubated for additional 1-2 h. Optical density was measured at 490 nm with a plate reader (Victor2, 1420 multilabel counter, Perkin-Elmer).

6 Migration assays

For wound-healing assays C2C12 myoblasts transfected with nestin RNAi oligos were seeded on 12-well plates and cultured until confluent monolayer. Scratch wounds were done using a pipette tip and washed with DMEM containing 1% FBS to remove loosely

held cells. Images were captured immediately after washing and following an overnight incubation. Transwell migration assays were performed using 6.5 mm Transwell inserts (Corning Incorporated) coated with polycarbonate membrane with 8.0 µm pores. The protocol was adapted from the study by Ödemis and coworkers (2007). For migration assay 50 000 control or nestin-depleted C2C12 myoblasts were placed in 100 µl of DMEM containing 1% FBS into the inner well of the insert while the outer well was filled with 600 µl of DMEM containing 1% FBS. Chemoattractant SDF-1α (PeproTech) was added to the outer well in a concentration of 10 ng/ml and the inserts were incubated for 5 h at 37°C. The cells that had not migrated through the membrane were wiped off using cotton stick and the migrated cells were fixed with 3% PFA for 10 min at RT. The membranes were carefully removed from inserts and mounted in Vectashield containing DAPI. Migrated myoblasts were counted with Leica DMRE microscope.

7 Protein analyses

7.1 SDS-polyacrylamide gel electrophoresis and Western blotting (I-III)

Soluble cell extracts were prepared by lysis in Laemmli sample buffer, subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane (Protran nitrocellulose, Schleicher & Schuell) using semidry or wet transfer apparatus (Bio-Rad, GE Healthcare). Membranes were blocked with 5% non-fat milk in PBS containing 0.3% Tween-20 for 1 h at RT. Western blotting was performed using antibodies against nestin (BD Pharmingen), pThr-316 nestin (Sahlgren *et al*., 2003), vimentin (BD Pharmingen and V9, Sigma), GFP (JL-8, Living Colors), p35 (C-19 and N-20, Santa Cruz Biotechnology and P9489, Sigma), Cdk5 (DC34, Biosource Invitrogen and C-8, Santa Cruz Biotechnology), PKCξ (Santa Cruz Biotechnology), poly(ADP-ribose) polymerase (PARP; Sigma), p21 (C-19, Santa Cruz Biotechnology), p27 (C-19, Santa Cruz Biotechnology), myogenin (M-225, Santa Cruz Biotechnology), troponin T (JLT-12, Sigma), MHC (H-300, Santa Cruz Biotechnology), desmin (RD301, Santa Cruz Biotechnology), Ki-67 (Abcam), proliferating cell nuclear antigen (PCNA; Sigma), calpain 1 (Cell Signaling Technology), calpain 3 (Abcam), RhoA (Santa Cruz Biotechnology), 14-3-3τ (Santa Cruz Biotechnology), Hsc70 (SPA-815, Stressgen), and actin (AC-40, Sigma). After washes with PBS containing 0.3% Tween-20 membranes were probed with horseradish peroxidase-conjugated secondary antibodies purchased from Promega, Southern Biotechnology and GE Healthcare. Proteins were visualized using ECL detection kit (GE Healthcare).

7.2 Protein fractionation assays (I, III)

To analyze the subcellular localization of p35 as a function of nestin ST15A cells were transfected either with p35 alone or together with nest-640. Cells were harvested and lysed in Triton X-100 containing fractionation buffer (25 mM Hepes [pH 7.6], 100 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, 0.5% Triton-X 100, complete protease inhibitor mixture [Roche]) for 30 min on ice. Cell lysates were separated into insoluble and soluble pools by centrifugation at 15 000 x *g* for 40 min at 4°C. The supernatant and the pellet fractions, the latter containing highly insoluble cytoskeletal and membrane-bound proteins were resuspended in Laemmli sample buffer and analyzed by Western blotting. HeLa and C2C12 cells transfected with nest-640 were fractionated in an equal manner.

ST15A cells were transfected with plasmids encoding p35 and Cdk5 together with either nest-640 or empty GFP and incubated with 100 μM H_2O_2 for 6 h. To prepare nuclear enriched fractions harvested cell pellets were carefully suspended in 800 μl of buffer containing 10 mM Tris (pH 7.4), 10 mM NaCl, 3 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM dithiothreitol (DTT), and complete protease inhibitor mixture and incubated for 10 minutes on ice. Following incubation 0.3% of NP-40 was added to each sample and the tubes were inverted a few times. Incubation was continued for an additional minute after which the samples were centrifuged at 400 x *g* for 4 min at 4ºC. Pellets were carefully washed with the buffer described above and centrifuged again (400 x *g*, 4 min, 4ºC). Recovered pellets were suspended in 150 μl of buffer consisting of 20 mM Tris (pH 7.4), 40 mM $Na_4P_2O_7$, 5 mM $MgCl_2$, 50 mM NaF, 100 μM Na_3VO_4, 10 mM EDTA, 1% Triton-X 100, 1% SDS, and protease inhibitors, sonicated briefly and centrifuged at 20 000 x *g* for 10 min at 4ºC. Supernatants comprising now the nuclei were suspended in Laemmli sample buffer and subjected to Western blotting.

7.3 Coimmunoprecipitation analyses (I-III)

To identify the Cdk5 interaction site on nestin, ST15A cells transfected with GFP-tagged nestin constructs were subjected to Cdk5 immunoprecipitation. Cells were harvested, suspended in lysis buffer (20 mM Hepes [pH 7.4], 140 mM NaCl, 10 mM pyrophosphate, 5 mM EDTA, 0.4% NP-40, 100 mM PMSF, complete protease inhibitor mixture) and kept on ice for 30 min. Lysates were lightly sonicated and centrifuged at 10 000 rpm for 10 min at 4ºC. The supernatants were precleared with protein A-sepharose beads (Sigma), followed by the immunoprecipitation of Cdk5 with polyclonal anti-Cdk5 antibody (C-8, Santa Cruz Biotechnology) under rotation overnight at 4ºC. Immunocomplexes were captured with 50% slurry of protein A-sepharose at 4ºC on a rotamix for 4 h. The beads were collected with mild centrifugation, washed twice with lysis buffer and twice with PBS containing complete protease inhibitor mixture and suspended in Laemmli sample buffer.

For analyzing the association between nestin and the Cdk5/p35 signaling complex during oxidative stress ST15A cells were transfected with nest-640, p35 and Cdk5 and treated with H_2O_2. At indicated time points, medium containing dead cells was discarded and living cells still attached to the plate were lysed in buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.05% SDS, 5 mM EDTA, 5 mM EGTA, and complete protease inhibitor mixture on ice for 30 min. Lysates were centrifuged at 15 000 x *g* for 10 min at 4°C and the supernatants were precleared with protein A-sepharose. Lysates were incubated with anti-p35 C-19 and N-20 antibodies (Santa Cruz Biotechnology) in shaking overnight at 4ºC. Immunocomplexes were precipitated with protein A-sepharose on a rotamix for 2 h at 4ºC and washed three times with the lysis buffer. Immunoprecipitated proteins were eluted from the beads by boiling in Laemmli sample buffer, run on SDS-PAGE and detected by Western blotting. The analysis of the interaction between nest-640 and endogenous Cdk5 during C2C12 differentiation was performed using the same coimmunoprecipiation protocol with the exception that the cleared cell lysates were immunoprecipitated with polyclonal anti-GFP antibody (Living Colors, BD Biosciences) for 1 h at 4°C under rotation followed by the capture of immuno-complexes by protein A-sepharose beads for 4 h at 4°C.

To asses the effect of nestin on p35 ubiquitylation, ST15A cells were transfected with His-tagged p35, HA-tagged ubiquitin together with either nest-640 or empty GFP vector and treated with or without 20 μM MG132. Cells were lysed in lysis buffer containing 25 mM Hepes (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 20 mM β-glycerophosphate, 20 mM para-nitro-phenyl phosphate, 1 mM PMSF, 1 mM DTT, complete protease inhibitor mixture, 5 μM MG132 and 20 mM N-ethylmaleimide. Ubiquitylated p35 was precipitated with protein G-sepharose beads (Amersham Biosciences) pre-coupled with anti-HA antibody (Santa Cruz Biotechnology).

C2C12 and Cos7 cells were subjected to immunoprecipitation with anti-p35 and anti-PKCξ (Santa Cruz Biotechnology) antibodies in lysis buffer consisting of 50 mM Hepes (pH 7.4), 140 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, 0.4% NP-40, 10 mM pyrophosphate, 5 mM Na_3OV_4, and complete protease inhibitor mixture. Cells were lysed for 30 min on ice and the particulate material was centrifuged at 12 000 x *g* for 10 min at 4°C. Protein concentration was measured by the Bradford assay and 800 μg of protein per lysates was precleared using sepharose beads. The cleared lysates were incubated with above mentioned antibodies and the immunocomplexes were captured with protein G-sepharose beads and washed four times in 20 mM Hepes (pH 7.4), 2 mM EGTA, 100 mM NaCl, 0.4% NP-40, and 1 mM DTT. Beads were finally resuspended in Laemmli sample buffer, boiled and analyzed by Western blotting.

For immunoprecipitation of the Cdk5/p35 complex cell pellets were lysed on ice in immunoprecipitation buffer suitable for the Cdk5/p35 interaction analysis (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 1 mM PMSF, complete protease inhibitor mixture) for 30 min. Insoluble material was centrifuged at 15 000 x *g* for 10 min at 4°C after which samples were taken for input control. Lysates were precleared with protein A-sepharose for 1 h at 4°C and then incubated with anti-p35 antibody (C-19, Santa Cruz Biotechnology) for 1 h at 4°C in a rotamix. Protein A-sepharose beads were added and the incubation under rotation was continued for additional 4 h at 4°C. Sepharose beads were washed three times with immunoprecipitation buffer, resuspended in Laemmli sample buffer and the immunoprecipitated proteins were analyzed by Western blotting.

7.4 Kinase activity assays (I, II)

Cdk5 kinase activity was analyzed from H_2O_2-treated ST15A cells lysed in buffer containing PBS (pH 7.4), 1% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM PMSF, 0.5 mM DTT, and complete protease inhibitor mixture for 30 min on ice. To obtain a crude whole cell extract, samples were homogenized 8 times with 26 G needle and centrifuged at 14 000 rpm for 15 min at 4°C. The protein concentration was determined using the Bradford assay and the samples were normalized accordingly. Cdk5 was immunoprecipitated with anti-Cdk5 antibody (C-8, Santa Cruz Biotechnology) and pulled down with protein A-sepharose beads. Beads were washed twice with lysis buffer and twice with kinase buffer (50 mM Mops [pH 7.4] and 5 mM $MgCl_2$). The immunocomplexes were resuspended in 20 μl of kinase buffer containing histone H1 (100 μg/ml) as a kinase substrate and 1.2 μl of ATP mix (2 μl of γ-^{32}P ATP in 28 μl of 2 mM cold ATP in kinase buffer) was added to start the reactions. Samples were incubated at 30°C for 20 min. The reactions were stopped by the addition of Laemmli sample buffer and boiling for 5 min. The samples were further centrifuged at

12 000 × *g* for 5 min at RT and run on SDS-PAGE. Phosphate incorporation into histone H1 was visualized by autoradiography. To analyze the Cdk5 activity in soluble fraction, ST15A cells were transiently transfected with p35 and nestin constructs, collected and lysed in fractionation buffer (25 mM Hepes [pH 7.6], 100 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, 0.5% Triton-X 100, complete protease inhibitor mixture) for 30 min on ice. Lysates were centrifuged at 15 000 x *g* at 4°C for 40 min and the supernatants were collected as soluble fractions. Immunoprecipitation of Cdk5 and kinase reaction were performed as described above.

To measure PKCξ and Cdk5 activity during myoblast differentiation, PKCξ and Cdk5 were immunoprecipitated from myoblasts harvested at indicated timepoints. Cells were lysed in buffer containing 50 mM Hepes (pH 7.4), 140 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA, 0.4% NP-40, 10 mM pyrophosphate, 5 mM Na_3OV_4, and complete protease inhibitor mixture. Lysates were centrifuged at 12 000 x *g* for 10 min at 4°C and precleared with protein G-sepharose beads prior to immunoprecipitation with anti-PKCξ and anti-Cdk5 (C-8, Santa Cruz Biotechnology) antibodies in a rotamix at 4°C overnight. Following an incubation with protein G-sepharose beads the immunoprecipitates were collected by mild centrifugation and washed four times with 50 mM Hepes (pH 7.4), 5 mM EDTA, 125 mM NaCl, 0.2% NP-40, and 1 mM DTT and twice with 50 mM Hepes (pH 7.4), 25 mM NaCl, and 1 mM DTT. For PKCξ activity assay beads were resuspended in PKCξ kinase buffer (50 mM Hepes [pH 7.4], 1 mM EDTA, 1 mM DTT, 10 mM $MgCl_2$) and for Cdk5 activity assay in Cdk5 kinase buffer (50 mM Tris-HCl [pH 7.4], 5 mM $MgCl_2$, 1mM EDTA, 1 mM DTT). Histone H1 (3 μg) together with 200 μM ATP and 10 μCi of γ-^{32}P ATP was added to each sample. The reactions were allowed to proceed for 30 min at 32˚C (PKCξ) and at 25˚C (Cdk5) and were stopped by the addition of Laemmli sample buffer and boiling for 5 min. Samples were run on SDS-PAGE and subjected to autoradiography.

7.5 *In vitro* protein phosphorylation and phosphorylation site identification (II)

In vitro phosphorylation assays were performed using immunoprecipitated p35, His-tagged p35 N-terminus (amino acids 1-120, Bioclone Inc), GST-tagged p35 C-terminus (amino acids 208-308, Abnova), the His-Cdk5/GST-p35 complex (Abnova), immunoprecipitated calpain 3 and recombinant calpain 1 (Calbiochem) as substrates. Immunoprecipitations were performed as described with PKCξ activity assay. The *in vitro* phosphorylation reactions were done in PKCξ kinase buffer (50 mM Hepes [pH 7.4], 1 mM EDTA, 1 mM DTT, 10 mM $MgCl_2$). 50 ng of recombinant human PKCξ (Upstate Biotechnology) together with 200 μM ATP and 10 μCi of ^{32}P-ATP was added to each sample. The reactions were allowed to continue for 30 min at 32˚C and were stopped by the addition of Laemmli sample buffer and boiling. SDS-PAGE was subjected to autoradiography.

In order to identify the PKCξ-specific phosphorylation sites on p35, His-tagged human p35 purified from Sf9 insect cells (Sakaue *et al*., 2005; Yamada *et al*., 2007) was used as a substrate in *in vitro* reaction where 3 μg of the recombinant p35 protein was incubated with or without 10 ng of recombinant human PKCξ in a buffer consisting of 50 mM Tris-HCl (pH 7.5), 0.1% 2-mercaptoethanol, 0.1 mM EGTA, 0.1 mM sodium vanadate, 10 mM magnesium acetate, and 100 μM γ-^{32}P ATP for 30 min at 30°C. Kinase

reactions were stopped by boiling in Laemmli sample buffer and the samples were resolved by SDS-PAGE and subjected to autoradiography. p35 bands were excised, reduced with DTT, alkylated with iodeacetamide, and then in-gel digested with trypsin. The tryptic digests were analyzed by liquid chromatography-tandem mass spectrometry using an LTQ-Orbitrap XL mass spectrometer (Xcalibur 2.0.7 software; Thermo Fisher Scientific) coupled to EASY-nLC (Proxeon). PepMap C18 trap column (0.3 x 5 mm; LC-Packings) and PepMap C18 analytical column (0.075 x 150 mm; LC-Packings) were used.

7.6 Metabolic ^{32}P-labeling and phosphopeptide mapping (II)

HEK293 cells were transfected with constitutively active PKCξ together with either WT or S33A-mutated myc-tagged p35 and let to recover for 24 h. The recovered, transfected cells were preincubated in phosphate-free minimum essential medium Eagle (MEME, Sigma) supplemented with 10% FBS, 2 mM L-glutamine and antibiotics for 30 minutes prior to the labelling with 0.3 mCi/ml ^{32}P-orthophosphate (PerkinElmer) in MEME for 3 hours. Cells were harvested, rinsed with PBS and subjected to p35 immunoprecipitation. Cell pellets were lysed in immunoprecipitation buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.05% SDS, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 0.1 mM $NaOV_3$, 0.5 mM PMSF, 30 mM NaF, complete protease inhibitor mixture) for 30 min followed by centrifugation at 15 000 x *g* for 10 min at 4°C. Lysates were precleared with 50% slurry of protein A-sepharose beads for 1 h at 4°C and the cleared cell lysates were immunoprecipitated with anti-p35 antibody (C-19, Santa Cruz Biotechnology) under rotation overnight at 4°C. Immunocomplexes were captured with protein A-sepharose beads for 4 h at 4°C, washed three times with immunoprecipitation buffer and resuspended in Laemmli sample buffer. Samples were separated by SDS-PAGE followed by autoradiography with Fujifilm BAS-1800 Bioimaging analyzer.

In-gel tryptic digestions of ^{32}P-labeled WT and S33A-mutated p35 followed by 2D phosphopeptide mapping were carried out as previously described (Kochin, *et al.*, 2006). The labeled p35 bands were excised from the dried gel and in-gel digested overnight at 37°C with 2 ng/µl sequencing grade trypsin (Promega) in 50 mM ammonium bicarbonate buffer (pH 8). Supernatants were collected, vacuum-dried and resuspended in 15 µl of pH 1.9 buffer (formic acid 2.3%, acetic acid 2.9%). Peptides were then separated in two dimensions by electrophoresis and thin layer chromatography (TLC). For the first dimension 15 µl of the tryptic digest was applied on a cellulose sheet (20 cm x 20 cm, Merck KgaA) and electrophoretically separated in pH 1.9 buffer at 750 V for 1.5 h using the Hunter Thin Layer Peptide Mapping System (C.B.S. Scientific). The sheet was dried and ascending TLC in the second dimension was performed for 13 h in a chromatography tank saturated with a mobile phase containing 30% water, 37.5% n-butanol, 7.5% acetic acid, and 25% pyridine. The sheet was dried and the ^{32}P phosphopeptides were visualized by autoradiography.

7.7 Calpain assays (II)

The brains of 2-day-old FVB-n mice were isolated and homogenized in lysis buffer (20 mM Hepes [pH 7.4], 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA), using a Teflon grinder (Sato *et al*., 2007). Each homogenate was centrifuged at 13 000 x *g* for 25 min at 4˚C. Pellets were resuspended in 100 µl of lysis buffer, sonicated, and centrifuged again (13 000 x *g*, 25 min, 4˚C.). Recovered supernatants were divided into control and actual sample. Calpain activity was induced by the addition of 4 mM $CaCl_2$ and the samples were incubated for 1 h at 37˚C. The reaction was stopped by the addition of EGTA to a final concentration of 10 mM and boiling in Laemmli sample buffer. C2C12 myoblasts transfected with WT and S33A myc-tagged p35 were treated equally to induce calpain activity. The processing of p35 was analyzed by Western blotting.

Following a PKCξ-induced phosphorylation described above (without 10 µCi of ^{32}P-ATP), the His-Cdk5/GST-p35 complex was subjected to calpain cleavage assay using a commercial kit according to the manufacturer's instructions (QIA120, Calbiochem). After incubation with or without recombinant calpain 1 for 5 min at RT, the reactions were stopped by the addition of Laemmli sample buffer. Samples were separated by SDS-PAGE and analyzed by Western blotting. Commercial luciferase-based assay (calpain-Glo assay, Promega) was used to monitor the calpain activity in myoblasts upon PKCξ inhibition. C2C12 cells were allowed to differentiate for 72 h in the presence or absence of either PS inhibitor (20 µM) or calpain inhibitor III (15 µM) and the activity of calpains was measured in each sample using the kit according to the manufacturer's protocol.

8 Statistics (I-III)

Viability of H_2O_2-treated ST15A cells transfected with mock, dnCdk5, nest-1177, nest-640, or cotransfected with nest-1177 and Cdk5, was analyzed by MTT assay and the results were presented as relative cell viability as related to the control (untreated population at 0 h) of each transfection. The graph represented the mean values of six experiments (*P<0.05). The comparisons of C2C12 differentiation and p35 degradation were made using GraphPad Prism software. In differentiation analyses the amount of MHC-positive cells was counted from 3-6 independent experiments and the results were presented as relative differentiation. For statistical analysis of the proliferation of nestin-depleted myoblasts, values representing the relative proliferation were obtained from five independent experiments. The comparison of WT and S33A p35 processing to p25 was presented as normalized p25/p35 ratio. Quantification was made from the Western blots of three independent experiments using densitometry (ScionImage GelPlot 2). Likewise the quantification of p35 processing in SW13 cells was made from the Western blots of three independent experiments using densitometry and the obtained values were normalized to untreated control samples (value 1). Statistical significance was analyzed with unpaired Student's *t*-tests for data from nestin overexpressing myoblasts whereas paired Student's *t*-tests were performed for nestin downregulation and p35 degradation data and was marked by asterisks (***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$ and ns, $P > 0.05$). The bar graphs represent mean values and the standard errors of mean.

RESULTS AND DISCUSSION

1 Nestin scaffold regulates the Cdk5/p35 signaling complex during oxidative stress (I)

During the past decade IFs have emerged as regulatory scaffolds for a diverse range of signaling molecules (Pallari and Eriksson, 2006b). This intriguing ability has revolutionized the view on IF functions rendering IFs from solely mechanical proteins to active regulators of signal transduction. Earlier work from our group had described an association between the Cdk5/p35 signaling complex and IF protein nestin in progenitor cells of CNS, in differentiating myoblasts and at the NMJs (Sahlgren *et al*., 2003). We were tempted to scrutinize the role of nestin as a regulator of Cdk5/p35 and, thereby to further broaden the view on IFs as multifunctional signaling scaffolds. To execute our plans we needed a model system.

Reactive oxygen species (ROS), such as free radicals and peroxides, are molecules that emerge due to metabolism of oxygen. Most of cellular ROS derives from normal metabolic reactions, for example oxidative phosphorylation, but also environmental pollutants and radiation can increase the amount of ROS. Since excess of ROS is detrimental for organisms, cells have efficient enzymes, the most well-known ones being superoxide dismutase, catalase, and glutathione peroxidase, to detoxify these reactive intermediates and to maintain the normal reducing environment within the cells. Oxidative stress arises from the imbalance in the generation and inactivation of ROS. Free radicals of oxygen are highly reactive and can damage all cellular components including proteins, lipids and DNA, subsequently leading to apoptotic cell death. Oxidative stress is a common pathogenic factor also in several neurodegenerative diseases (reviewed by Barnham *et al*., 2004). Neuronal progenitor cells have evoked interest due to the fact that they could provide a possible treatment for neurodegenerative disorders by means of regenerative therapy. Therefore, to know how neuronal progenitor cells behave in pathological environment and what kind of molecular mechanisms they exploit in responding to excess of ROS would increase the understanding of neuronal progenitor cell biology. Considering the death promoting activity of Cdk5 upon oxidative stress (Strocchi *et al*., 2003, Sun KH *et al*., 2008) and our findings regarding the association between nestin and the Cdk5/p35 signaling complex in progenitor cells of CNS (Sahlgren *et al*., 2003) we decided to address the functional importance of nestin as a regulator of Cdk5/p35 upon the exposure of neuronal progenitors to H_2O_2.

1.1 Proteasome-mediated downregulation of nestin is associated with the induction of apoptosis upon oxidative stress (I)

We exposed the ST15A neuronal progenitor cells to H_2O_2 to induce oxidative stress. Consistently with the current understanding of oxidative stress as a mediator of apoptotic cell death (Ozben, 2007), the H_2O_2-treatment of ST15A cells triggered apoptosis. This was evidenced by morphological changes, cell shrinkage and rounding, chromatin condensation visualized by DAPI staining (Figure 1A, I) and DNA fragmentation detected with PI flow cytometry assay (Supplementary figure 1, I). Most importantly, we observed a rapid downregulation of nestin, as already at 5-6 hours of H_2O_2-treatment nestin was

nearly completely depleted (Figure 1A, I). The downregulation of nestin seemed to precede the execution of apoptosis since at the time of nestin downregulation the cleavage of PARP or vimentin, both of which are known targets of caspase-3 (Boulares *et al*., 1999, Buyn *et al*., 2001), was not evident.

Disassembly of the cytoskeleton is characteristic to apoptotic cell death and several IF proteins including vimentin, desmin and K18 are implicated as caspase targets (Caulin *et al*., 1997, Buyn *et al*., 2001, Chen *et al*., 2003). We found interesting differences both in the kinetics and mechanisms of downregulation of coexpressed vimentin and nestin forming heteropolymers. Firstly, the depletion of nestin occurred fast and was rather complete by the time when the cleavage of vimentin was induced and the protein amount of vimentin started to decline (Figure 1A, I). When examining the detached (apoptotic) cells, both the intact and caspase-cleaved vimentin were present whereas the full length nestin was almost totally absent from apoptotic cells further exemplifying the different kinetics of downregulation (Figure 1B, I). Similar differences between nestin and vimentin downregulation was observed also when programmed cell death was triggered in ST15A cells with the topoisomerase II inhibitor etoposide as well as in PC3 and DU145 prostate cancer cells with TRAIL (data not shown). Secondly, the downregulation of nestin appeared not to be dependent on caspase activity as the general caspase inhibitor Z-VAD was not able to prevent the nestin downregulation (Figure 1C, I). Instead, nestin depletion seemed to originate from proteosomal activity since the treatment with the proteasome inhibitor MG132 efficiently blocked the downregulation of nestin. The proteasome pathway has been stated to govern the rapid reduction of nestin also during neuronal differentiation (Mellodew *et al*., 2004), a discovery in line with our results.

1.2 Nestin is a survival determinant during oxidative stress (I)

Considering our observations alluding to the downregulation of nestin as a prerequisite for the execution of cell death, we were eager to test whether the RNAi-mediated depletion of nestin prior to the exposure to H_2O_2 could affect the sensitivity of ST15A neuronal progenitor cells. Indeed, the depletion of nestin clearly sensitized the cells to oxidant-induced apoptosis as indicated by the increased amount of apoptotic cells among nestin-depleted cells compared to untransfected control cells or cells transfected with scrambled vector (Figure 2B, I). This result was further verified by using MTT cell viability assay and analyzing the PARP cleavage (Figure 2C-D, I). Contrary to the nestin silencing, the overexpression of nestin had a protective effect (Figure 3, I). The incidence of apoptosis as indicated by chromatin condensation was significantly lower in nestin overexpressing cells than in cells overexpressing empty GFP-vector. Thus, the rapid downregulation of nestin appears influential for the execution of oxidant-induced cell death. This result is in agreement with numerous studies describing enhanced cell death upon IF depletion (for example Caulin *et al.*, 2000, Gilbert *et al*., 2001, 2004, 2008, Jaquemar *et al*., 2003, Tong and Coulombe, 2006), thereby corroborating the rather evident survival-promoting functions of IFs. Intriguingly, the antiapoptotic functions of nestin were recently highlighted in a study where the injection of nestin morpholino into zebrafish eggs resulted in developmental defects in the brain and eyes including reduced head and brain size as well as small and poorly organized eyes (Chen HL *et al*., 2010). These abnormalities in embryo development turned out to derive from increased neuronal progenitor cell apoptosis. Hence, the concept of nestin as a potent survival determinant has important ramifications for understanding the regulation of cell survival,

not only when neuronal progenitor cells are exposed to stress but also during neuronal development.

1.3 Cdk5 activity is regulated by nestin during oxidative stress (I)

After establishing that nestin is downregulated during H_2O_2-induced apoptosis we wanted to confirm that Cdk5 is activated in this model system and determine whether Cdk5 activation correlates with nestin downregulation. Both the expression of Cdk5 activator protein p35 and Cdk5 activity started to increase 5 hours after the induction of H_2O_2-treatement reaching the maximum at 6 hours (Figure 4A, I). By this time nestin was almost completely downregulated demonstrating that the elimination of nestin occurs before the maximal Cdk5 activity is obtained.

As IFs have been determined to regulate a large number of signaling determinants (Pallari and Eriksson, 2006b) we wanted to delineate whether the interaction between nestin and Cdk5 could explain the nestin-mediated modulation of oxidant-induced cell death. Indeed, we detected an increase in Cdk5 activity in nestin-depleted cells upon H_2O_2-treatment compared to control cells transfected with scrambled vector (Figure 4B, I). This indicates that the nestin scaffold could affect the progress of H_2O_2-induced apoptosis by regulating the activation of death promoting Cdk5. Moreover, the expression of dnCdk5 in nestin-depleted cells reversed the sensitizing effect of nestin downregulation, hence suggesting that nestin is upstream of Cdk5 in the signaling cascade leading to oxidant-induced apoptosis (Figure 4C, I). However, the RNAi-mediated nestin downregulation did not lead to spontaneous Cdk5-dependent apoptosis, although low levels of Cdk5 and p35 were estimated to be released from the IF scaffold to the cytoplasm (Figure 2B, I). This implies that oxidative stress provides an additional activation stimulus for Cdk5. In fact, an induction in the expression of Egr-1, a transcription factor regulating the expression of p35, is associated with oxidative stress and, hence, the enhanced Cdk5 activity is suggested to derive from the increased Egr-1-mediated expression of p35 (Strocchi *et al*., 2003).

In our previous study we had observed a colocalization of Cdk5 along nestin filaments in differentiating myoblasts (Sahlgren *et al*., 2003). The interaction was further confirmed by coimmunoprecipitation analysis of nestin and Cdk5/p35 from both C2C12 myoblasts and ST15A neuronal progenitor cells (Sahlgren *et al*., 2003). To investigate the Cdk5-binding region on nestin in more detail, we utilized a series of nestin deletion mutants: nest-314, consisting of the short N-terminus and α-helical rod domain, nest-640 and nest-1177, two constructs with increasing length of the C-terminal tail as well as nest-T332, corresponding to the last 332 amino acids of the nestin C-terminus. These mutants differed in, besides their assembly properties, but also in their ability to associate with Cdk5 (Figure 5, I). According to the coimmunoprecipitation assays Cdk5 interacted with nest-640 and nest-1177 but not with the deletion mutants representing the central rod domain or the far C-terminal domain, nest-314 and nest-T332 respectively (Figure 5D and Supplementary figure 5, I). This result places the Cdk5-binding region (amino acids 315-640) to the C-terminal domain of nestin. Compared to the endogenous nestin, nest-640 appeared remarkably more stable during oxidative stress (Figure 5C, I). This was an interesting observation suggesting that the far C-terminal region of nestin is involved in determining nestin degradation. We were eager to investigate how these more stable nestin truncations, nest-640 and nest-1177, capable to sequester Cdk5, modulate the oxidant-induced apoptosis in ST15A neuronal

progenitor cells. We observed that the cells overexpressing nestin deletion mutants were less prone to apoptosis induced by H_2O_2-treatment (Figure 6, I). To illustrate that the protective effect was due to the deregulation of Cdk5 we did an experiment where we compared the viability of nest-1177 transfected cells to nestin-1177/Cdk5 double transfected cells after incubation with H_2O_2. The increased viability of nest-1177 expressing cells was reversed by the simultaneous overexpression of Cdk5, pointing to the ability of nest-1177 to reduce the proapoptotic activity of Cdk5 (Figure 7A-B, I). Results were further strengthened by observations showing that nestin truncation nest-640, Cdk5 and p35 formed a complex in H_2O_2-treated ST15A cells (Figure 7C, I).

1.4 Nestin affects the subcellular localization, turnover and processing of p35 (I)

The results presented above indicated that the prosurvival role of nestin would reside from the sequestration of the Cdk5/p35 complex and attenuation of Cdk5 activity. To examine in more detail the nestin-mediated regulation of Cdk5 activity, we induced an imbalance in the equilibrium between soluble and polymerized nestin by elevating nestin expression. Nestin deletion mutant nest-640 is not completely incorporated into the preexisting IF network composed of endogenous nestin and vimentin heteropolymerizing at a molar ratio of 1:4 (Steinert *et al*., 1999), but a fraction of nest-640 remains soluble increasing the soluble nestin pool within the cells (Figure 5B, I). Therefore, we transfected ST15A cells with nest-640 and p35 to induce Cdk5 activation, fractionated the harvested cell lysates, and analyzed the distribution of p35 which served as an indicator of the localization of activated Cdk5. In the absence of nest-640, p35 was localized mainly to the detergent insoluble fraction containing the cytoskeleton and cell membrane (Figure 8A, I). Considering the interactions between the Cdk5/p35 complex and endogenous, mainly filamentous nestin (Sahlgren *et al*., 2003) as well as the myristoylation-induced targeting of p35 to the cell membrane (Patrick *et al*., 1999, Kusakawa *et al*., 2000), this result was rather presumable. However, the transfection of nest-640 shifted p35 from the pelletable to soluble fraction. Moreover, Cdk5 activity was more intense in the detergent soluble pool when p35 was cotransfected with partially soluble nest-640 (Figure 8B, I). These results demonstrate the pronounced effect of nestin on the subcellular localization of p35 and, thereby, corroborate our assumption of the nestin-mediated sequestration of the Cdk5/p35 signaling complex.

Different regulatory mechanisms governing Cdk5 activity have been described in the literature. For example, the activity of Cdk5 is regulated by a negative feedback loop where the Cdk5-mediated phosphorylation of p35 results in ubiquitylation and proteosomal degradation of p35 (Patrick *et al*., 1998). As nestin seemed to efficiently modulate the Cdk5/p35 complex through sequestration we wanted to determine whether the interaction between nestin and p35 could affect the turnover of p35. We transfected ST15A cells with nest-640, p35 and HA-tagged ubiquitin and treated the cells with the proteasome inhibitor MG132. It appeared that p35 was significantly less ubiquitylated when nestin was overexpressed (Figure 9A, I) indicating that nestin-mediated sequestration promotes the stability of p35. Consistently with this discovery, the half-life of p35 was prolonged upon the overexpression of nestin deletion mutant (Figure 9B, I).

Another well-defined mechanism regulating the Cdk5 activity is the calpain-mediated cleavage of p35 to a more stable activator, p25, occurring during various neurotoxic conditions (Strocchi *et al*., 2003, Wang et al., 2003, Tang *et al*., 2005, Saito *et al*., 2007).

The generation of p25 leads to deregulated Cdk5 activity with detrimental effects. We detected an increase in the generation of p25 that correlated with nestin depletion and Cdk5 activation in terms of kinetics (Figure 7D, I) Therefore, we made an attempt to determine whether the nestin scaffold could have an effect on the cleavage of p35. We found that the overexpression of nestin in the form of nest-640 restrained the generation of p25 in stressed cells suggesting that the interaction with nestin protects p35 from calpains (Figure 7D, I). Taken together, nestin-mediated sequestration of p35 appears to efficiently modulate the fate of p35 by protecting it from different modes of proteolytic processing.

What is then the mechanism underlying the nestin-mediated regulation of p35? The anchorage of p35 to nestin IFs could reduce the susceptibility of p35 to calpain attack due to sequestration of p35 away from the activated calpains or steric masking of the potential calpain-cleavage sites on p35. Interaction-based protection of the calpain target proteins is not unusual and, for example Rb has been illustrated to be protected from calpain-mediated cleavage by the association of serine protease inhibitor SerpinB2 to the calpain cleavage site of Rb (Tonnetti *et al*., 2008). The cytoprotective role of SerpinB2 in TNFα-induced apoptosis was shown to stem from the elevated levels of Rb repressing the transcription of proapoptotic genes through a negative regulation of E2F family of transcription factors. On the other hand, the Cdk5-dependent phosphorylation of T138 on p35 has been described to reduce the susceptibility of calpain-mediated p35 cleavage (Kamei *et al*., 2007). This was considered to derive either from the reduced affinity of calpains or selective binding of other proteins to T138-phosphorylated p35. Hence, alternatively, the p35 interaction with nestin could modulate the phosphorylation status of p35 or nestin could sequester the phosphorylated form of p35. Moreover, the phosphorylation of T138 was observed to facilitate the proteosomal degradation of p35, thereby explaining the decreased probability to become exposed to calpains (Wei *et al*., 2005a, Kamei *et al*., 2007). On the basis of our results it could be estimated that the sequestration of phosphorylated p35 by nestin would both interfere with the targeting of phosphorylated p35 to proteasomes, thereby increasing the half-life of p35, and hamper the calpain-mediated generation of death-promoting p25. Phosphorylation of T138 on p35 has been proposed to function as a regulatory switch and the resistance of fetal p35 to calpain-dependent cleavage to derive from the increased phosphorylation on this particular site (Kamei *et al*., 2007). In adult brain where different forms of stress can enhance the generation of p25, the levels of phosphorylated T138 were significantly reduced. Therefore, exploring the relationship between nestin expression and the phosphorylation of p35 on T138 as well as the ability of nestin to sequester phosphorylated p35 would reveal whether these regulatory mechanisms cooperate in protecting the developing CNS from uncontrolled Cdk5 activity.

Due to the lack of an N-terminal myristoylation group, p25 is more prominently distributed to the cytosol and nucleus, thereby enabling spatially less restricted Cdk5 activation (Patrick *et al*., 1999, Kusakawa *et al*., 2000). Increased nuclear localization of p25 has been observed in neurons in which cell death is triggered by ER stress (Saito *et al*., 2007), glutamate-induced excitotoxicity or by treatment with a DNA-damaging agent (O'Hare *et al*., 2005). O'Hare and colleagues presented that the Cdk5/p25 complex would promote cell death within the nucleus whereas the cytoplasmic Cdk5/p35 complex would mediate prosurvival signals. In support of their conclusion, Cdk5 is known to modulate the activities of nuclear proteins. For example, survival factor MEF2 is inactivated by Cdk5 (Gong *et al*., 2003, Tang *et al*., 2005) whereas Cdk5 stimulates the cell cycle regulator Rb causing an aberrant and detrimental cell cycle reentry (Hamdane

et al., 2005). We sought to define the effect of nestin on the subcellular localization of p35/p25 during oxidative stress. ST15A cells were transfected with plasmids encoding p35 together with either GFP-tagged nestin deletion mutants or empty GFP-vector as a control, incubated with H_2O_2 and stained with anti-p35 antibody recognizing both p35 and p25. Nestin deletion mutants turned out to sequester p35 to the cytoplasm as the cytoplasmic labeling of p35 was clearly decreased in the presence of empty GFP-vector (Figure 7E, I). Respectively, p25 accumulated in the nucleus of control cells transfected with empty GFP-vector as demonstrated by the immunostaining and Western blot analysis of nuclearly enriched fractions (Supplementary figure 6, I).

In conclusion, in the utilized model system where neuronal progenitor cells were exposed to oxidant-induced cell death, the enhanced Cdk5 activity and the appearance of p25 correlated with the induction of apoptosis. The protective effect of dnCdk5 (Figure 7A, I) indicates that Cdk5 has functional significance and that the generation of p25 is not only a mere consequence of apoptotic protease activity. Furthermore, the cleavage of p35 to p25 is known to change the subcellular distribution of activated Cdk5 and allow the phosphorylation of aberrant target proteins. Thus, the accurate regulation of the Cdk5/p35 signaling complex appears extremely essential. We observed that ectopic expression of IF protein nestin remarkably promoted cell survival in neuronal progenitor cells exposed to oxidative stress. This was proposed to reside from the nestin-mediated sequestration of the Cdk5/p35 signaling complex into the cytoplasm and the ability to increase the stability and decrease the processing of p35 to death-promoting p25 (Figure 14). Therefore, nestin can be concluded to function as an important regulator of the Cdk5/p35/p25-mediated signaling pathway and the survival-promoting function of nestin to stem from the regulation of apoptosis-modulating signaling (section 2.1.2). The downregulation of nestin appears as an essential step in the cascade of events leading to the activation of Cdk5 and the induction of apoptosis during oxidative stress.

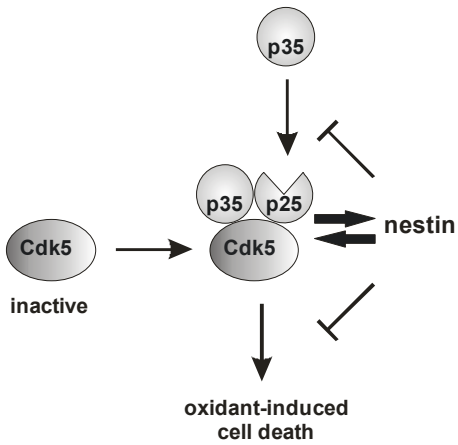

Figure 14. Nestin-mediated regulation of the Cdk5/p35/p25 signaling complex during oxidative stress. Cdk5 becomes activated during oxidative stress either by p35 or its cleavage product p25. (To simplify the scheme Cdk5, p35 and p25 are presented here in one complex, although Cdk5 interacts with one activator at a time.) Nestin protects neuronal progenitor cells from oxidative stress-induced cell death through the sequestration of the Cdk5/p35 signaling complex into the cytoplasm and through the ability to increase the stability and decrease the processing of p35 to death-promoting p25. In addition, Cdk5 participates in the regulation of its own nestin scaffold (discussed in section 3.1).

1.5 Does nestin scaffold regulate the Cdk5/p35 complex in other physiological contexts?

As the Cdk5 activity is restricted to certain cell types and cellular activities, observations of nestin as a regulator of the Cdk5/p35 complex raise the following questions: Does

nestin expression typify the cell types undergoing Cdk5-mediated cellular processes? Can the neural nestin-mediated regulation of Cdk5 be extended to other cell types and distinct cellular events? Indeed, following our observation describing the interplay between nestin and the Cdk5/p35 complex in apoptotic neuronal progenitor cells, the modulatory function of nestin over Cdk5 has been referred to in other cell systems. RNAi-mediated depletion of nestin has been presented to render vascular smooth muscle cells more prone to H_2O_2-induced apoptosis (Huang YL *et al*., 2009b). However, in these cells Cdk5 seemed to play a prosurvival role and phosphorylate Bcl-2 which is known to enhance the antiapoptotic potential of Bcl-2 (Cheung *et al*., 2008). The stimulation of stressed vascular smooth muscle cells with EGF induced nestin expression and promoted cell survival, the effect assumed to arise from the regulation of Cdk5 (Huang YL *et al*., 2009b). Nestin and Cdk5 have been shown to colocalize also in kidney podocytes, the epithelial cells of renal glomerulus, where Cdk5 was suggested to regulate the IF cytoskeleton in podocyte processes (Bertelli *et al*., 2007). Moreover, the interrelationship between nestin and Cdk5 could be hypothesized to play a regulatory function also in other contexts. Concomitant expression of nestin and Cdk5 is apparent in testicular Leydig cells (Lin *et al*., 2009, Lobo *et al*., 2004) as well as in neuronal progenitors launched to migrate from the ventricular zone of developing cortex (Clarke, 2007). The latter indication prompts to postulate that nestin could play a role in determining the early induction and pace of Cdk5-dependent neuronal migration. Recently both nestin and Cdk5 have been described to be essential for the migrational ability of prostate cancer cells (Kleeberger *et al*., 2007, Strock *et al*., 2006). Moreover, considering the specific accumulation of nestin and Cdk5 in growth cones (Nikolic *et al*., 1996, Yan *et al*., 2001), the extensions of developing axons searching synaptic targets, it is even more tempting to speculate that nestin would participate in the regulation of Cdk5/p35 during cell motility. Taken together, the expression patterns of nestin and Cdk5 appear seemingly similar and, hence, the ability of nestin to operate as an organizational scaffold for Cdk5/p35 in multiple tissues and cell compartments during a variety of cellular functions seems conceivable. In the future, careful examination of the upcoming nestin KO mice could provide important *in vivo* evidences how/if the various Cdk5-dependent events in different tissues are affected by nestin.

2 PKCξ acts as an upstream regulator of Cdk5 in differentiating myoblasts (II)

Skeletal muscle differentiation is a multistep process entailing the irreversible withdrawal of proliferating myoblasts from the cell cycle as well as the expression of muscle-specific genes and culminating to the fusion of myoblasts to multinucleated myotubes (Buckingham, 2006, Shi and Garry, 2006, Grefte *et al*., 2007). Myogenesis is tightly regulated and characterized by the activation of a vast number of signaling determinants ranging from cell surface molecules to transcriptional regulators and cytoplasmic signaling modulators (reviewed by Jansen and Pavlath, 2008). Among others, Cdk5 has been implicated as a regulator of myogenesis (Lazaro *et al*., 1997, Philpott *et al*., 1997). We have earlier demonstrated the formation of a Cdk5-nestin signaling complex in myoblasts and shown that Cdk5 regulates the reorganization of nestin filaments during myogenic differentiation (Sahlgren *et al.*, 2003). However, relatively little is known about the upstream regulation of Cdk5 during myogenesis. In differentiating L6 rat myoblasts the PI3K-Akt-p70S6K pathway has been presented to promote Cdk5 activity by upregulating the transcription factor Egr-1, known to mediate the induction of p35

expression (Sarker and Lee, 2004). We wanted to further explore the signaling cascades essential for myogenic differentiation and investigate the mechanisms of Cdk5 activation in differentiating myoblasts in more detail. In the light of the information of PKCξ in development (for example Wooten *et al*., 1994, 1999, Mansat-De Mas *et al*., 2002, Martin *et al*., 2002, 2005), we pursued to study whether PKCξ acts in myogenesis.

2.1 Progress of myoblast differentiation is critically regulated by PKCξ (II)

To asses the possible role of PKCξ in myogenic differentiation, we downregulated PKCξ using RNAi or inhibited PKCξ activity by employing a specific PKCξ pseudosubstrate (PS) inhibitor both in mouse primary myoblasts and in C2C12 myoblast cell line. The PS inhibitor is a synthetic peptide corresponding to the PKCξ PS sequence. It is linked to a myristoyl group to facilitate the transport through the cell membrane. This peptide suppresses the activity of PKCξ by an autoinhibitory mechanism by associating to the substrate-binding cavity of PKCξ catalytic domain. The specificity and the effective dose of PS inhibitor have been extensively verified in several studies (Sun *et al*., 2005, Fishelevich *et al*., 2006). Scrambled peptide with identical myristoyl group was utilized to exclude the unspecific effects of the myristoyl group and peptide treatment.

Myoblasts were induced to differentiate and the progress of differentiation was analyzed by detecting the formation of multinucleated myotubes and the presence of the differentiation markers MHC, desmin and troponin. The downregulation of PKCξ (siPKCξ) in primary myoblasts efficiently abrogated myoblast fusion and the expression of myogenic markers when compared to the cells transfected with scrambled RNAi oligos (Figures 1A and 2A, II). Similarly, the PS inhibitor efficiently blocked myoblast differentiation without any toxic effects. The formation of multinucleated myotubes was impaired and the expression of myogenic markers reduced following the PS treatment both in primary (Figures 1B and 2B, II) and C2C12 myoblasts (Supplementary figures 1A-B, II). Differentiation of myoblasts was not affected in the presence of Calphostin C, an inhibitor of conventional and novel PKCs (Supplementary figure 1C, II), thereby confirming the specific and essential function of atypical PKCξ in myoblast differentiation.

2.2 PKCξ is a master controller of Cdk5 activity during myogenic differentiation (II)

By examining the protein levels and the activation of PKCξ during myoblast differentiation we aimed at to analyze in more detail the role of PKCξ in this process. While the protein levels of PKCξ remained relatively stable during the differentiation process (Figure 3A, II), the activity of PKCξ was significantly increased at 48 hours of differentiation (Figure 3B, II). In addition to our research group, Ödemis and coworkers (2007) have examined and reported the effects of PKCξ in differentiating myoblasts. However, they utilized a different model system as they investigated the potential effects of stromal derived factor 1 (SDF-1) on the migration and differentiation of myoblasts. Chemokine SDF-1 and its receptor, CXC chemokine receptor 4 (CXCR4), have been extensively studied in hematopoietic cells but have also been reported to affect limb myogenesis (Ödemis *et al*., 2005). Ödemis and coworkers (2007) observed that the

treatment of myoblasts with SDF-1 induced the activation of PKCξ and promoted the ability of myoblasts to migrate but attenuated differentiation. Inhibition of PKCξ abolished the inhibitory effect of SDF-1 on the induction of differentiation. The results by us and Ödemis and coworkers besides demonstrate the critical role of PKCξ in myogenesis but also highlight the importance of the strict temporal regulation of PKCξ activity.

We paid also attention to the kinetics of PKCξ activation which correlated with the previously investigated Cdk5 activity (Sahlgren *et al*., 2003). Thus, we were eager to explore the possible crosstalk between PKCξ and Cdk5. To address whether the inhibition of Cdk5 reduces PKCξ activity, we treated the differentiating C2C12 myoblasts with the Cdk5 inhibitor Roscovitine. However, no difference was detected in the PKCξ activation although Cdk5 inhibition efficiently blocked the progress of differentiation (Supplementary figure 2, II). As this result indicated that PKCξ acts upstream of Cdk5 in the differentiation process, we proceeded to investigate the effect of PKCξ inhibition on Cdk5 activity. The PS-treatment resulted almost total inhibition of Cdk5 activity (Figure 4A, II) which we interpreted to confirm our hypothesis of PKCξ lying upstream of Cdk5 in the signaling cascade directing the differentiation of myoblasts.

2.3 PKCξ promotes the calpain mediated processing of p35 to p25 (II)

While searching the mechanism of PKCξ to stimulate Cdk5 activity, we tested the ability of PKCξ to directly interact with p35 in myoblasts. We were able to point out that the association between PKCξ and p35 was rather weak in undifferentiated myoblasts but increased when differentiation proceeded implying that the PKCξ-mediated regulation of Cdk5 activity could stem from the regulation of p35 (Figure 5B, II). Prompted by this result we surveyed whether PKCξ phosphorylates p35 and found that, indeed, p35 was a substrate of PKCξ (Figure 5C, II). The stretch of p35 targeted by PKCξ was narrowed down by two different recombinant fragments of p35 utilized in *in vitro* phosphorylation assays with recombinant PKCξ. Only the N-terminal fragment of p35 turned out to be phosphorylated by PKCξ (Figure 5D, II). For us this experiment provided interesting information, as it showed that p35 is phosphorylated on the N-terminus which is cleaved and removed to give rise to the p25 activator. Later on S33 on p35 was verified as a PKCξ-specific phosphorylation site with mass spectrometry and *in vivo* phosphorylation analyses (Supplementary figures 4-5, II).

Although Cdk5 has been implicated to be important for skeletal muscle development (Lazaro *et al*., 1997, Philpott *et al*., 1997), the mechanisms of Cdk5 and p35 regulation have not been thoroughly analyzed. While exploring the effect of PS-treatment on the protein levels of p35 during myoblast differentiation, we detected an appearance of a protein band the size of which appeared to be around 25 kDa (Figure 6A, II). This band was recognized by the C-19 anti-p35 antibody that specifically interacts with the p35 C-terminus. The calpain-mediated cleavage of p35 to p25 is well established in neurodegenerative diseases where the hyperactivation of Cdk5 by p25 leads to damaging phosphorylation events (Patzke and Tsai, 2002a, Cruz and Tsai, 2004). Therefore, the possible occurrence of p25 during myoblast differentiation was a major surprise for us and we invested a lot of work to convince ourselves about the identity of p25. In addition to confirming the dependence of p25 generation on calpain activity (Figure 6B, II), we reproduced an experiment described in the literature where the $CaCl_2$-

treatment of brain lysates induces the cleavage of p35 and the generation of p25 (Sato *et al*., 2007). We were able to show that the p25 produced in $CaCl_2$-treated mouse brain lysates matches with the p25 produced during the differentiation of mouse primary myoblasts (Supplementary figure 6B, II). The treatment of differentiating myoblasts with the calpain inhibitor revealed that besides inhibiting the generation of p25, calpain inhibitor blocked the differentiation process (Figure 6B, II). This result supports the physiological relevance of p25 as an effector of Cdk5 during myogenic differentiation. Despite the prominent neurodegenerative functions, p25 has been depicted also in other non-detrimental physiological processes. Moderate amounts of p25 have been described to positively modulate the signal transduction at synapses and improve cognitive functions (Fischer *et al*., 2005, Ris *et al*., 2005). However, the putative role of Cdk5/p25 in normal homeostasis emphasizes the need for a tight spatiotemporal regulation of p35 processing.

The experiments that uncovered the generation of p25 during the differentiation of C2C12 and primary myoblasts indicated also an involvement of PKCξ in the process as the PS inhibitor significantly decreased the formation of p25 (Figures 6A and 6C, II). Thus, the obvious question arising was, how PKCξ affects the calpain-mediated processing of p35. Cdk5-mediated phosphorylation of T138 on p35 has been proposed to negatively regulate the susceptibility of p35 to calpain-dependent cleavage (Kamei *et al*., 2007). Therefore, we decided to investigate if the PKCξ-specific phosphorylation site, S33 on p35, affects the calpain-mediated processing of p35. We performed site-directed mutagenesis to replace S33 with an alanine residue and studied the susceptibility of the mutated p35 to calpain-mediated processing by utilizing two well-established methods where the calpain activation is triggered either by the calcium ionophore A23187 or by $CaCl_2$ (Kusakawa *et al*., 2000, Kamei *et al*., 2007). The treatment of C2C12 myoblasts transfected with WT p35 or p35 S33A with A23187 or the treatment of transfected cell lysates with $CaCl_2$ followed by Western blot analysis revealed that p35 S33A is more resistant to calpain-mediated cleavage than WT p35 (Figure 8A and Supplementary figure 8, II). Moreover, an *in vitro* cleavage assay where the Cdk5/p35 complex was pre-incubated with the recombinant PKCξ prior to the incubation with the recombinant calpain 1 confirmed that the cleavage of p35 to p25 was more prominent following the PKCξ-dependent phosphorylation (Figure 8B, II). Our results were consistent with the existing literature showing that the inhibition of Cdk5 by Roscovitine increases the generation of p25 in *in vitro* cleavage assays (Kamei *et al*., 2007). However, we observed that the PKCξ-mediated regulation of p35 processing was not affected by Cdk5 but more likely PKCξ potentiates the proteolytic degradation of p35. This illustrates the capability of PKCξ to override the Cdk5-dependent autoinhibitory loop. Based on our data we hypothesized that the PKCξ-dependent phosphorylation of p35 on S33 is a regulatory switch enabling the fast spatiotemporal formation of p25 in differentiating myoblasts. The mechanistic details, whether the PKCξ-mediated phosphorylation of p35 to S33 directs p35 to calpains or promotes the calpain-dependent cleavage by preventing the protecting phosphorylation events (other than Cdk5-dependent), remain to be determined.

While studying the role of PKCξ-dependent phosphorylation on the calpain-mediated p35 processing, we tested also the ability of PKCξ to affect the p35 cleavage by directly regulating calpain activity. The identification of another aPKC, PKCι, as a regulator of calpains (Xu and Deng, 2006) prompted us to test this hypothesis. We observed that the inhibition of PKCξ activity by the PS inhibitor or downregulation of PKCξ expression by

RNAi decreased the autocleavage (indicating activation) of both ubiquitous calpain 1 (μ-calpain) and muscle-specific calpain 3 in C2C12 and mouse primary myoblasts (Figures 7A-B, II). Moreover, according to a luciferase-based calpain activity assay, the inhibition of PKCξ resulted in an overall decrease in calpain activity at 72 hours of differentiation (Supplementary figure 7A, II).

Ca^{2+}-dependent intracellular proteases, calpains have been previously depicted to have a critical regulatory role during the different stages of myogenic differentiation. Calpain activation has been described to regulate myoblast migration critical for the early steps of muscle development (Leloup *et al*., 2007). The overexpression of endogenous calpain inhibitor, calpastatin, has been reported to prevent myoblast fusion and inhibit the degradation of talin (Barnoy *et al*., 1998, 2005), the proteolysis of which is important for adhesion site disassembly and turnover (Franco *et al*., 2004). Specific downregulation of calpain 2 diminished the adhesiveness and inhibited the fusion of C2C12 myoblasts (Honda *et al*., 2008). The importance of calpains in myogenesis has been examined also *in vivo*. Specific disruption of *calpain 3* gene in mice resulted reduced muscle mass (Kramerova *et al*., 2004). More elaborate analysis of the calpain 3 KO myoblasts revealed a disturbed sarcomere organization although myoblast fusion occurred rather normally. Later on the membrane-bound M-cadherin/β-catenin complex was stated to be cleaved by calpain 3 but, intriguingly, this modification appeared to regulate myogenesis by preventing the excess of myoblast fusion (Kramerova *et al*., 2006). Calpains have also been shown to target signaling determinants such as PKCα in differentiating myoblasts (Liang *et al*., 2006). Our results delineate an interesting role for PKCξ at the outset of calpain activation in differentiating myoblasts. Moreover, we managed to show that calpain 3 interacts with PKCξ during differentiation and is a direct phosphorylation target of PKCξ (Supplementary figure 7B-C, II). Whether PKCξ-dependent regulation governs also other calpains or different cellular activities marked by calpain activation would be fascinating to study.

In summary, we provide data from the requirements for p25 during myogenic differentiation and depict PKCξ as the major upstream regulator of the Cdk5/p35/p25 signaling pathway in differentiating myoblasts (Figure 15). PKCξ is suggested to function both by phosphorylating p35 into a calpain cleavage-permissive form and by boosting calpain activity. Although PKCξ KO mice are available for research purposes, there are no reports, however, applying the involvement of PKCξ with muscle development. In fact, the mouse models for PKCξ and also for Cdk5 have been characterized in detail only in terms of lymphoid organs (Leitges *et al*., 2001, Martin *et al*., 2002) and CNS (Ohshima *et al*., 1996, 1999, Gilmore *et al*., 1998) respectively. In the light of our results, the specific reexamination of the skeletal muscles and myocardium in PKCξ KO mice would be of concern, although *in vivo*, however, it is worth remembering that the other aPKC, the ubiquitously expressed PKCλ, could compensate for the loss of ζ isoform (Martin *et al*., 2002). Furthermore, considering the potential beneficial role of Cdk5/p25 in non-pathological CNS processes, such as learning and memory (Fischer *et al*., 2005, Ris *et al*., 2005, Angelo *et al*., 2006), it would be captivating to analyze what kind of mechanism could define the strictly regulated generation of p25. Brain-specific atypical protein kinase M ζ (PKMζ) is generated from mRNA transcribed from an internal promoter within the PKCζ gene (Hernandez *et al*., 2003). Thus, the sequence of PKMζ contains the C-terminal catalytic domain of PKCξ without the N-terminal regulatory region due to which PKMζ is autonomously active. Interestingly, PKMζ activity is associated with Cdk5/p25-dependent processes. It is involved in the maintenance of

LTP at synapses, which is critical for synaptic plasticity and memory formation (Hernandez *et al*., 2003, Yao *et al*., 2008). In addition, PKMζ is observed to accumulate to neurofibrillary tangles in the neurons of limbic system in AD (Crary *et al*., 2006). Although being less abundant in CNS, also PKCζ has been implicated important for neurite elongation, a process utilizing Cdk5 (Mori *et al*., 2009). Therefore, studying the significance of aPKCs in the regulation of p35 during neuronal development and homeostasis as well as during neurodegenerative disorders could perhaps provide novel information of the p35 processing in CNS.

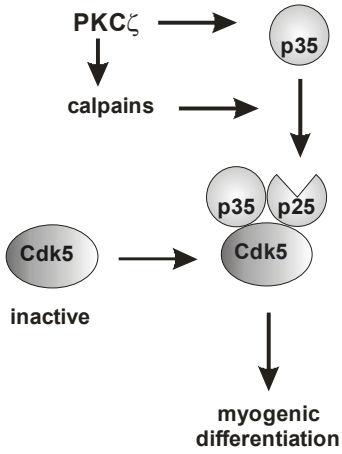

Figure 15. PKCζ acts as a master controller of Cdk5 activity in differentiating myoblasts. PKCζ regulates the activity of Cdk5 by enhancing the calpain-mediated processing of p35 to a more stable activator, p25, through p35 phosphorylation and calpain activation.

2.4 Putative functions of Cdk5/p25 during myogenesis

When deliberating the potential functions of the Cdk5/p25 complex during myogenesis one often encounters the fact that the formation of p25 generally depicts apoptotic responses (O'Hare *et al*., 2005, Saito *et al*., 2007). However, myogenic differentiation has been described to possess several features of apoptosis. Matrix metalloproteinases, the activation of which triggers programmed cell death, appear essential for the myotube formation (Yagami-Hiromasa *et al*., 1995). Caspase-3 activation is required for myoblast fusion and, moreover, those caspase-3 KO mice that survive to early perinatal life show a reduction in skeletal muscle mass compared to WT littermates (Fernando *et al*., 2002). In addition, the overexpression of apoptosis repressor with caspase recruitment domain inhibits myoblast differentiation (Hunter *et al*., 2007). Therefore, one could assume that the functions and target proteins of the Cdk5/p25 complex in differentiating myoblasts would resemble those identified in stressed neurons. For example, the Cdk5-mediated phosphorylation of the transcription factor MEF2 incurs the caspase-dependent MEF2 degradation, thereby impairing the ability of MEF2 to protect neurons from apoptosis (Gong *et al*., 2003, Tang *et al*., 2005). Consistently, the Cdk5/p25-dependent phosphorylation of MEF2, central factor also in differentiating myoblasts (Naya and Olson, 1999), could perhaps adjust the timing and progression of myogenesis.

The generation of p25 in differentiating myoblasts could also reflect the need for prominent although rather acute Cdk5 activity as well as the less restricted subcellular distribution of activated Cdk5. It has been reported that the nuclear localization of Cdk5 defines the postmitotic state of neurons (Zhang *et al*., 2008). Thus, keeping in mind the

nuclear localization of p25 (Patrick *et al*., 1999, Kusakawa *et al*., 2000), it would be tempting to postulate that the PKCξ-mediated formation of p25 would enable the nuclear activity of Cdk5, thereby prohibiting differentiating myoblasts from reentering the cell cycle. However, it was recently reported that the Cdk5-dependent cell cycle suppression in neuronal cells derives from the association between Cdk5/p35 complex and transcription factor E2F1 in the nucleus (Zhang *et al*., 2010). This interaction, reducing the DNA binding ability of the cell cycle regulator E2F1, was observed to be dependent on p35 but not p25. Among nuclear proteins, the activities of which are facilitated by Cdk5, are also p53 and Rb (Hamdane *et al*., 2005, Lee *et al*., 2007). Interestingly, both p53 and Rb have been established important for myoblast differentiation the latter being implicated in irreversible cell cycle withdrawal and in the expression of late muscle differentiation markers (Porrello *et al*., 2000, De Falco *et al*., 2006). Although claiming the importance of p25 during myogenesis seems justified based on the introduced studies, to fully understand the role of p25 during myogenesis requires further experimentation.

3 Nestin stability and organization are regulated by Cdk5 (I, II, III)

Phosphorylation-based regulation critically determines the dynamic nature of IF proteins (Omary *et al*., 2006, Pallari and Eriksson, 2006a). Both the assembly state and stability can be modulated by the phosphorylation of IF protein head and tail domains. In addition to functioning as a scaffold for the Cdk5/p35 complex, nestin has appeared to be prominently phosphorylated by Cdk5 (Sahlgren *et al*., 2003). From our earlier studies we knew that the Cdk5-mediated phosphorylation is essential for the disassembly and reorganization of nestin filaments upon myoblast differentiation (Sahlgren *et al*., 2003). In this PhD project I wanted to address whether Cdk5 affects also nestin turnover and stability.

3.1 Cdk5 affects nestin turnover in H_2O_2-treated neuronal progenitor cells (I)

Previous work from our laboratory had characterized T316 and T1495 in the nestin C-terminal domain as Cdk5-dependent phosphorylation sites (Sahlgren *et al*., 2003). In addition, we have got indications of two additional putative Cdk5 phosphorylation sites, S620 and S729, residing both in the nestin tail domain (Hanna-Mari Pallari and Vitaly Kochin unpublished observations). Taking into account this pronounced targeting of nestin by Cdk5, we decided to analyze if activated Cdk5 regulates its own scaffold in H_2O_2-treated ST15A neuronal progenitor cells. Inhibition of Cdk5 activity by Roscovitine or transfection with dnCdk5 reduced nestin degradation upon oxidative stress, indicating that Cdk5 is involved in the regulation of nestin turnover (Supplementary figure 3, I). The possible direct involvement of Cdk5 in regulating nestin degradation is consistent with the observation that the nestin deletion mutant, nest-640, lacking the established Cdk5 specific phosphorylation site T1495 (as well as the potential site S729) is significantly more stable during oxidative stress than the full length nestin (Figure 5C, I). According to these observations it seems that Cdk5-mediated phosphorylation may promote the degradation of nestin in stressed neuronal progenitor cells. As earlier indicated, despite the apparent nestin degradation and the generation of a nestin cleavage product during oxidative stress (Figure 1A, I), nestin is not proteolyzed by caspases. However, stress induces the activation of several proteolytic enzymes including calpains, the ability of

which to process nestin has not been investigated. Dephosphorylation of NFs has been reported to protect NFs from calpain-mediated proteolysis (Pant 1988, Greenwood *et al*., 1993) raising the questions whether also nestin could be a calpain target and what would be the significance of the Cdk5-mediated nestin phosphorylation in this mode of degradation.

3.2 PKCξ-Cdk5 signaling cascade determines nestin reorganization and stability in differentiating myoblasts (II, III)

An earlier piece of work by our research group had proven that Cdk5 is required for the reorganization of nestin filaments into parallel fibers during the fusion of C2C12 myoblasts into multinucleated myotubes (Sahlgren *et al*., 2003). Cdk5 phosphorylated nestin on T316, thereby promoting the disassembly of nestin filaments which was postulated to precede and enable the structural modifications in nestin IFs occurring during myoblast fusion. Therefore, while investigating the functions of PKCξ-Cdk5 signaling axis in differentiating myoblasts, we decided to use nestin as a physiological downstream marker for Cdk5 activity. We observed that in the presence of the PS inhibitor or when PKCξ was downregulated with RNAi, nestin reorganization was severely disturbed in differentiating myoblasts (Figure 4B-C, II). The morphological abnormalities in cells corresponded with the previously observed modifications upon Cdk5 inhibition (Sahlgren *et al*., 2003) and further confirmed that PKCξ controls myogenic differentiation through the regulation of Cdk5. During our attempts to characterize the PKCξ downstream target proteins we tested also the ability of PKCξ to phosphorylate IF proteins. Interestingly, using IF preparation isolated from ST15A neuronal progenitor cells as a substrate, we noticed that PKCξ efficiently phosphorylated vimentin but not nestin (data not shown, Hanna-Mari Pallari and Aurelie de Thonel, unpublished observations). The result was further confirmed using bacterially expressed vimentin as a PKCξ substrate. In future it would be interesting to examine whether vimentin is phosphorylated by PKCξ also *in vivo* and whether this phosphorylation affects the vimentin filament structure. It is tempting to speculate that the Cdk5-mediated nestin disassembly would be accompanied by the PKCξ-dependent regulation of vimentin to accomplish the extensive IF reorganization characteristic to myoblast fusion. Furthermore, taking into account the described interaction between PKCξ and K10 (Paramio *et al*., 2001), it would be worth exploring whether the nestin/vimentin IFs control the PKCξ-dependent regulation of p35 through scaffolding.

In addition to the structural modifications, we decided to dissect the effect of the PKCξ-Cdk5 signaling pathway on nestin protein levels in differentiating C2C12 and primary myoblasts. Contrary to the stabilization of nestin upon Cdk5 inhibition in stressed neuronal progenitor cells, we discovered a remarkable decrease in nestin protein amount at 48 and 72 hours of differentiation when the PKCξ-Cdk5 signaling axis was inhibited by the PS inhibitor or PKCξ downregulation (Figure 4B-C, II, Figure 6B, III). In our previous work we did not detect as drastic changes in nestin protein amount following the Cdk5 inhibition (Sahlgren *et al*., 2003) but this could derive from experimental differences. Cdk5 inhibitor was applied on cells 24 hours before harvesting to focus on the effects of Cdk5 on nestin reorganization (Sahlgren *et al*., 2003) while the PS-treatment was initiated simultaneously with differentiation. Later on we confirmed that also long-term Cdk5 inhibition by Roscovitine or by transfection of dnCdk5 leads to decreased nestin protein amounts in differentiating myoblasts (Figure 6C, III). Hence,

the inhibition of the PKCξ-Cdk5 signaling cascade appears to have a prominent effect not only on nestin reorganization but also on nestin stability. Using an antibody recognizing the phosphorylated T316 on nestin C-terminus we detected stabilization in the amount of phosphorylated nestin during the late stages of myoblast differentiation when the overall nestin protein levels had started to decline (Figure 6A, III). This result is in agreement with the data from Cdk5 inhibition experiments and renders the Cdk5-dependent phosphorylation of nestin a critical determinant of nestin stability in differentiating myoblasts. In our previous report we observed that nestin phosphorylated on T316 is almost completely absent from the pelletable protein fraction containing filamentous IF proteins (Sahlgren *et al*., 2003). Therefore, the Cdk5-mediated phosphorylation of nestin upon myogenic differentiation could be hypothesized to protect the solubilized nestin from degradation while nestin filaments undergo reorganization.

In our experimental model systems, Cdk5 appeared to regulate the stability of nestin with opposing influences. Cdk5 had a degradation-promoting role in stressed neuronal progenitor cells but an apparent stabilizing effect in differentiating myoblasts. Since we have not so far achieved a complete understanding of the Cdk5-mediated phosphorylation of nestin concerning the phosphorylation sites and the specific effects of phosphorylation on each particular site, we can not rule out that there might be differences in the nestin phosphorylation profile and, therefore, in the outcome of Cdk5-mediated nestin phosphorylation in different circumstances. In addition, the phosphorylation of K8 on S74 has been depicted to protect K8 from ubiquitylation and proteasomal degradation (Ku *et al*., 2000) but in response to shear stress, the same phosphorylation site triggers the proteosomal degradation of K8 in alveolar epithelial cells (Jaitovich *et al*., 2008). These reports further emphasize the significance of the biological context in determining the role of posttranslational modifications and protein fate. Different biological processes trigger distinct signaling pathways indicating that, for example, the Cdk5-dependent regulation of nestin could be supplemented with context-specific signaling events participating in the determination of nestin stability.

3.3 Neuromuscular junction-associated signaling regulates nestin in a Cdk5-dependent manner

NMJs originate from the innervation of skeletal muscle. Agrin and NRG, released by the innervating neuron, have been implicated as important regulators of synaptogenesis controlling the organization of receptor clusters and NMJ specific gene expression respectively (Henriguez *et al*., 2008, Herndon & Fromm, 2008). Although nestin expression is downregulated in adult muscle, a portion of nestin remains specifically at the NMJs in an innervation-specific manner (Vaittinen *et al*., 1999). A close association between nestin and p35 is characteristic for NMJs alluding to the nestin-mediated scaffolding of Cdk5/p35 to junctions (Sahlgren *et al*., 2003). In fact, Cdk5 has been implicated to function at the NMJs. It is activated by NRG and critically regulates the activation of ErbBs, receptor tyrosine kinases through which NRG ensures the NMJ-specific gene expression (Fu *et al*., 2005). We decided to explore whether the synaptogenesis-mimicking signaling regulates nestin protein levels. Considering our results revealing the Cdk5-dependent nestin stabilization in differentiating myoblasts, we wanted to address also the possible role of Cdk5 in the accumulation of nestin at the NMJs. C2C12 myoblasts were induced to differentiate and fuse to myotubes and treated with agrin followed by a treatment with NRG. The results demonstrated an augmentation

in the protein amount of nestin upon NRG stimulus (Figure 16A). The accumulation of nestin was hampered by the Cdk5 inhibition (Figure 16B) suggesting that the agrin/NRG-signaling regulates nestin expression and stability at the NMJs in a Cdk5-dependent manner. Thus, it appears that Cdk5 could modulate its own targeting and activity by determining the nestin protein levels. Currently we are examining whether the effect of Cdk5 on nestin is due to the posttranslational modulation of nestin, affecting nestin stability, or stemming from an ErbB-induced increase in nestin expression. It seems that the increase in nestin protein levels is due to stabilization as RT-PCR analysis does not point to changes in nestin mRNA production (data not shown).

Cdk5 has also been depicted as being essential for the dispersion of AChR clusters that have not been innervated (Figure 10; Lin *et al*., 2005). Lin and colleagues (2005) presented a hypothesis that the Cdk5-mediated nestin phosphorylation leading to nestin reorganization could be a way to modulate the receptor organization, thus further underlining the dynamic interrelationship between nestin and Cdk5 at the NMJs. This hypothesis stems from the observation showing that due to the denervation of adult skeletal muscle nestin becomes spatially distributed from NMJs (Vaittinen *et al*., 1999), a phenomenon that could result from the phosphorylation-mediated reorganization of nestin. To summarize, the interplay between nestin and Cdk5 at the NMJs appears complex. Cdk5 may control its anchoring to NMJs through the regulation of nestin stability (Figure 16) but may also disperse the unwarranted receptor complexes by modulating the nestin organization (Lin *et al*., 2005). Thorough understanding of the different outcomes of Cdk5-dependent regulation of nestin at the NMJs requires a comprehensive insight into the effects of the Cdk5-mediated phosphorylation of nestin.

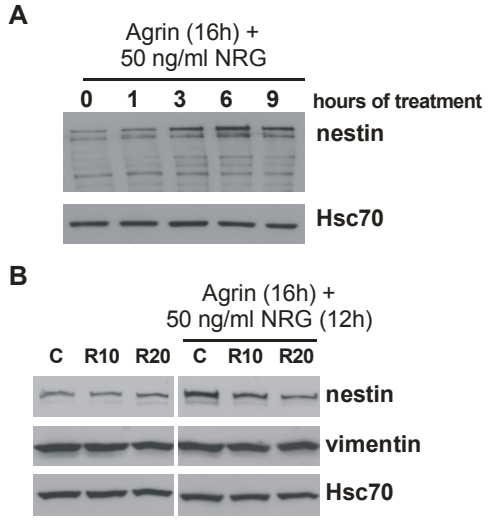

Figure 16. Agrin and NRG regulate nestin in a Cdk5-dependent manner. (A) C2C12 cells were induced to differentiate for 96 hours and treated with 10 ng/ml agrin overnight (16h) followed by a treatment with 50 ng/ml NRG for indicated times. Nestin protein levels were detected by Western blotting. Hsc70 was used as a loading control. (B) C2C12 cells were induced to differentiate for 120 hours and treated with 10 ng/ml agrin overnight (16h). After careful rinsing 10 or 20 µM Roscovitine was added to cells 30 min prior to the addition of NRG (50 ng/ml). Cell lysates were harvested 12 hours after NRG induction and subjected to Western blotting. Vimentin was used as a control to confirm the specific effect of agrin/NRG-treatment on nestin.

4 Onset and pace of myogenic differentiation are controlled by nestin (III)

Transient is perhaps the most apparent word to be used when describing the expression pattern of nestin. Nestin is present in stem and progenitor cells of various tissues but becomes downregulated and replaced by tissue specific IF proteins in the course of differentiation (Figure 17; Lendahl *et al*., 1990, Sejersen and Lendahl, 1993, Kachinsky *et al*., 1995, Fröjdman *et al*., 1997, About *et al*., 2000, Selander and Edlund, 2002, Chen *et al*., 2006, Bertelli *et al*., 2007). Due to this highly specific expression profile nestin could be assumed to define the general features of progenitor cells, such as increased viability, proliferation potential and capacity to migrate. In fact, as implicated in previous chapters, nestin is involved in protecting precursor cells from programmed cell death (Bieberich *et al.*, 2003, I), regulating the cell cycle progression (Reimer *et al*., 2009) and mediating the motile properties of cells (Kleeberger *et al*., 2007). An alternative function of nestin in developing tissues could be either to maintain the undifferentiated state of progenitor cells or to regulate the differentiation process itself, with special impact on timing, induction, and progression. According to our knowledge, the role of nestin in progenitor cell differentiation has not been addressed.

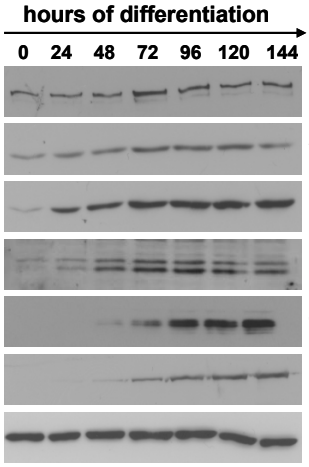

Figure 17. Dynamic expression pattern of nestin during the differentiation of C2C12 myoblasts. C2C12 myoblasts were induced to differentiate and samples for Western blot analysis were harvested at indicated time points. Nestin expression displays the characteristic pattern where the protein levels peak during the myotube formation at 72 hours of differentiation. Desmin, myogenin, troponin, and MHC were used to monitor the progress of differentiation. Hsc70 served as a loading control.

4.1 Downregulation of nestin accelerates myoblast differentiation (III)

We decided to utilize differentiating myoblasts as a model system to investigate the effect of nestin on cell differentiation. We downregulated nestin both in C2C12 myoblast cell line and in primary myoblasts derived from neonatal limb muscles. Myoblasts were induced to differentiate by serum withdrawal and the progress of differentiation was monitored. Downregulation of nestin was efficient and did not have any major effects on vimentin protein amounts (Figure 1C, III). Interestingly, nestin depletion appeared to have an effect on myoblast differentiation that was significantly accelerated. Nestin downregulated cells were more aligned and elongated compared to the cells transfected with scrambled oligos (Figure 1A, III). The cell cycle withdrawal of nestin-depleted cells was more efficient as indicated by the increased amounts of CKIs p21 and p27 (Figure 1B and Supplementary figure 2A, III). Furthermore, the expression of MRF myogenin as

well as the late myogenic markers troponin and MHC was clearly augmented (Figure 1C and Supplementary figure 2B, III).

The specific influence of cytoplasmic IF proteins on development has not been extensively studied but some interesting observations have been presented. Downregulation of muscle-specific IF protein desmin was indicated already years ago to have detrimental impact on myogenesis as the RNAi-based inhibition of desmin in C2C12 myoblasts perturbed differentiation (Li *et al*., 1994). Desmin KO mice are unable to maintain the proper muscle architecture and show severe disruption of muscle tissue with signs of degeneration increasing in the course of time indicating problems in muscle regeneration (Milner *et al*., 1996, Capetanaki *et al*., 1997). The most abundant glial cells, astrocytes, are known to modulate the differentiation of neuronal stem cells in neurogenic regions of adult brain. When neuronal stem cells were cocultured with astrocytes depleted of vimentin and GFAP, stem cells were observed to show enhanced neurogenesis suggesting that the IF network is elemental in determining the signaling cues provided by astrocytes (Widestrand *et al*., 2007). These findings, pointing to the role of IFs as regulators of differentiation, substantiate our data. The opposite outcomes of IF modulation in the presented examples, however, may be explained by the cell-specific functions of different IF proteins.

We would have been very eager to try to corroborate our results *in vivo* but, unfortunately, the nestin KO mice are not commonly available yet. IF protein vimentin, however, is implicated as a specific polymerization partner of nestin which alone is unable to form filaments (Steinert *et al*., 1999). Hence, we decided to isolate primary myoblasts from vimentin KO mice for differentiation analysis as previous studies have shown that the absence of nestin polymerization partner affects both nestin protein amount and assembly-state (Eliasson *et al*. 1999). We hypothesized that the ablation of vimentin would have consequences on nestin polymerization, thus affecting the mechanical and non-mechanical properties of nestin whereupon vimentin KO myoblasts could be used as an interim model for nestin effects. Indeed, we observed that the vimentin-deficient myoblasts were more prominently withdrawn from the cell cycle and, in a corresponding manner, showed elevated expression of the differentiation markers desmin and troponin (Figure 3A-B, III). Total nestin protein amount increased during differentiation but, contrary to our presumptions, there was no major difference in nestin protein levels between WT and KO myoblasts (Figure 3A, III). The ablation of vimentin has been reported to result a substantial decrease in nestin protein amount in primary astrocytes despite a considerable increase in nestin expression (Eliasson *et al*. 1999). This implies that the regulation of nestin in the absence of vimentin would be posttranslational. Therefore, our results showing rather equal amount of nestin both in WT and vimentin KO myoblasts could be interpreted to derive from the muscle specific posttranslational mechanism increasing the stabilization of nestin. The PKCξ-Cdk5 signaling axis could be postulated to answer for nestin stability during myoblast differentiation. Alternatively, the unaltered nestin protein levels could be explained by the ability of the existing IF protein desmin to stabilize nestin (Sjöberg *et al*., 1994b). If the latter option appears to be correct, it remains to be investigated whether nestin heteropolymerizes with desmin and how the putative nestin/desmin filaments would differ from nestin/vimentin structures especially in terms of non-mechanical properties. Nevertheless, our data clearly suggests that the modulation of either nestin protein amount or nestin filament structure affects the progress of myogenic differentiation.

The onset of myoblast differentiation provides an efficient cell cycle arrest and, therefore, increased differentiation is often associated with reduced proliferation rate. The cell cycle withdrawal and myogenic differentiation are regulated by a diverse range of intracellular signaling mechanism. For example, the attenuation of the leukemia inhibitory factor-stimulated JAK1–STAT1–STAT3 pathway inhibits myoblast proliferation and accelerates differentiation (Sun *et al*., 2007). Signaling launched by TGF-β significantly increases proliferation, thereby incurring a delay in differentiation (Schabort *et al*., 2009). Inspired by the surveys pointing to the importance of nestin for cell proliferation (Mokrý *et al*., 2004, Daniel *et al*., 2008, Huang YL *et al*., 2009a, Xue and Yuan, 2010) we sought to understand if the enhanced differentiation of nestin-depleted myoblasts correlated with the reduced proliferation rates. However, we did not detect any difference in cell proliferation between nestin-downregulated and control myoblasts measured by metabolic MTS assay (a measure of metabolic activity which in turn can be used as a cell number indicator) and the protein amounts of cellular proliferation markers Ki-67 and PCNA (Figure 1D-E, III). Moreover, nestin depletion did not lead to a precocious cell cycle withdrawal prior to the induction of differentiation with serum removal (Figure 1E, III). We also analyzed the migrational properties of nestin-deficient myoblasts as migration is critical for myoblasts to become dispersed in developing and regenerating muscle as well as to move towards their fusion partners (reviewed by Rochlin *et al*., 2010). Extracellular factors such as chemoattractants are implied to function as cues to myoblasts. SDF-1α and its receptor CXCR4 are associated with chemotaxis of a variety of cell types including myoblasts (Ödemis *et al*., 2007, Bae *et al*., 2008). However, we did not discern changes in the migratory response of nestin downregulated cells towards SDF-1α in Transwell migration assays compared to cells targeted with scrambled oligos (Figure 18). Neither scratch wound assays gave any difference between nestin-depleted and control cells (Figure 18). These results indicate that the main regulatory function of nestin is not to maintain the undifferentiated state of myoblasts or modulate the motility of myoblasts but relates to the differentiation process that has already been commenced. More specifically, nestin-depleted myoblasts do not become subjected to spontaneous differentiation but require additional differentiation-mediating cues from signaling related to serum deprivation and cell-cell adhesion.

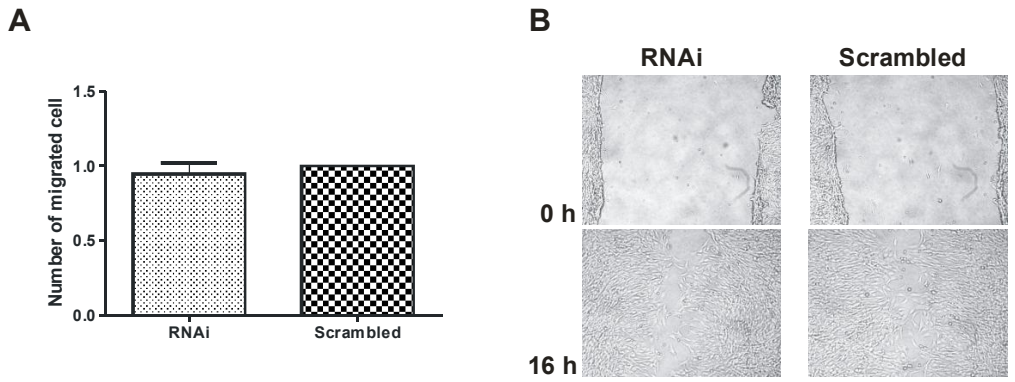

Figure 18. Downregulation of nestin does not affect the migrational abilities of undifferentiated myoblasts. (A) Nestin was downregulated in C2C12 myoblasts and the myoblasts were induced to migrate towards 10 ng/ml SDF-1α in Transwell inserts. Results are presented as relative cell migration and illustrated by GraphPad Prism. (B) Nestin-depleted C2C12 myoblasts were let to proliferate until reaching the confluence. Confluent monolayers were wounded using a pipette tip and images were captured at indicated time points to analyze the ability of myoblasts to migrate to wounded area.

To confirm our hypothesis about the role of nestin in determining the pace of myoblast differentiation we decided to make an attempt to delineate the reverse situation. To obtain stable levels of overexpressed nestin we transiently transfected C2C12 myoblasts with the N-terminal nestin deletion mutant containing the first 640 amino acids. The manipulation of myoblasts by the overexpression of nestin had a dramatic effect on differentiation as it almost completely inhibited the differentiation process (Figure 2, III). Cell cycle withdrawal was less efficient among nest-640 transfected myoblasts and the expression of myogenic differentiation markers remained low (Figure 2C-D, III). These analyses further strengthen the concept of nestin as a regulator of myoblast differentiation.

Studies aimed at delineating the cellular functions of nestin have been hampered by the lack of nestin KO mouse. This mouse model could be utilized to further investigate the role of nestin during besides embryonic but also injury-related regenerative myogenesis. The adult myogenesis is stated to differ from embryonic muscle development in terms of the gene expression profile of satellite cells and the physiological environment i.e. niche where the satellite cells differentiate (Lepper *et al*., 2009, Wang and Conboy, 2010). Moreover, as vimentin, desmin and GFAP, all coexpressed with nestin, are suggested to have a prominent effect on regenerative processes (reviewed by Capetanaki *et al*., 1997, Ivaska *et al*., 2007, Pekny and Lane, 2007), it would be worthwhile to assess the progress of satellite cell differentiation and the restoration of muscle architecture as well as how the muscle strength is recovered in injured muscle depleted of nestin. Despite the impaired regeneration, the ablation of vimentin or GFAP does not appear to disturb development (Colucci-Guyon *et al*., 1994, Pekny *et al*., 1999). It is interesting to see whether the same paradigm, more or less normal development but unfavorable outcome of regeneration, is repeated in nestin KO mice. Furthermore, what comes to the possible other functions of nestin, the features of programmed cell death have been related to myogenesis (Fernando *et al*., 2002). Therefore, an obvious question arising is whether nestin operates as a survival factor also in myogenic precursor cells similarly than in neuronal progenitors (I). More generally, considering the expression patterns of nestin being mainly restricted to progenitor cells, it would be intriguing to investigate whether the functions of nestin in developmental/regenerative processes are widespread and dissect whether nestin plays a role also in neuronal differentiation.

4.2 Nestin depletion increases the p35 processing in differentiating myoblasts (III)

Hitherto we have revealed that the interaction between nestin and the Cdk5/p35 complex is dynamic and suggested that there might be a Cdk5-dependent turnover of p35 on nestin scaffold in myoblasts induced to differentiate (Sahlgren *et al*., 2003). As a continuation on our efforts to delineate the role of nestin as a Cdk5/p35 scaffold we decided to assess the influence of nestin on the cleavage of p35 to p25 that was proven critical for the sustained Cdk5 activity in differentiating myoblasts (II). In neuronal progenitor cells exposed to Cdk5-dependent apoptosis, the overexpression of nest-640 was observed to have a survival promoting effect due to the sequestration of the Cdk5/p35 signaling complex and diminished generation of the more stable activator protein p25 (I). We decided again to elevate nestin expression with nest-640 and disturb the normal equilibrium between soluble and filamentous nestin in myoblasts. Consistently, we observed that nest-640, while inhibiting myoblast differentiation, sequestered Cdk5 (Figure 4C, III) and caused clear perturbations in the formation of p25

under differentiation promoting conditions (Figure 5A, III). Conversely, upon nestin silencing the amount of p25 generated was considerably increased arguing that the nestin scaffold regulates the cleavage of p35 to p25 (Figure 5B, III).

We determined to follow in more detail the Ca^{2+}/Calpain-dependent processing of p35 in the presence of nest-640 and came to a conclusion that most likely the small pool of nest-640 remaining soluble is responsible for protecting p35 from calpains (Figure 5C-E, III). We surmised that the soluble nest-640 facilitates the formation of the Cdk5/p35 complex through sequestration more efficiently than the filamentous nestin, thereby attenuating the p35 processing. Given these results and the increased differentiation of vimentin KO myoblasts, it could be speculated that nestin residing in vimentin KO cells is perhaps not entirely soluble but instead forms structures which, however, are unable to control the Cdk5/p35 complex and, thereby differentiation. In conclusion, our data depicts nestin as a potent effector of the Cdk5/p35/p25 signaling pathway and strengthens our primary findings presenting nestin as a key determinant affecting the p25 generation (I). The expression of nestin could be postulated critical for controlling physiological, non-detrimental cellular events requiring the p25-boosted Cdk5 activity by limiting the processing of p35. In addition, these results point to the power of soluble IF pool over signaling determinants, a subject that has not been extensively explored. Next step in our studies is to resolve whether the nestin-mediated sequestration of p35 in differentiating myoblasts regulates the PKCξ-dependent phosphorylation of p35 or protects the phosphorylated p35 form calpain attack or, alternatively, does the protection of p35 stem from a regulatory mechanism still unidentified.

4.3 Nestin and Cdk5 cooperate in promoting differentiation (III)

The substantial influence of nestin on the p35 processing indicated that nestin and Cdk5 function in the same pathway promoting differentiation. We sought to unravel this question by inhibiting Cdk5 in nestin-depleted myoblasts. Indeed, we were able to reverse the accelerating effect of nestin downregulation by the overexpression of dnCdk5 or inhibiting Cdk5 activity with Roscovitine (Figures 4A-B, III). The progress of differentiation was verified by immunoblotting myogenin, troponin and MHC. This observation together with the results showing the dependence of nestin stability and reorganization on the PKCξ-Cdk5 signaling cascade support a model where Cdk5 operates both upstream and downstream of nestin. While nestin elevation is dependent on Cdk5 activity, nestin will, once elevated, affect the activity of Cdk5 (Figure 19). Thus, the role of nestin in differentiating myoblasts could perhaps be to tightly regulate the accuracy of myoblast differentiation by affecting the timing, location and intensity of Cdk5 activity. Based on our data, we suggest that a strict PKCξ-Cdk5-dependent control of nestin stability and polymerization in fusing myoblast is essential as nestin appears to have a pronounced effect on the Cdk5/p35 complex and differentiation.

The profound regulatory effect of nestin on the induction of myogenesis can be observed already during the very early phases of differentiation. Therefore, it seems evident that, in addition to Cdk5, nestin could influence redundant signaling pathways. Myogenesis is regulated by a diverse range of signaling events and it is not hard to envision nestin as a recognized protein scaffold with precisely controlled expression pattern during myoblast differentiation to be involved also in other signal transduction pathways. The long C-terminal domain of nestin could be pictured to interact with signaling proteins similarly

than the long tail domain of synemin which has been described to directly associate with structural proteins, α-actinin, vinculin and talin, in muscle tissue (Bellin *et al*., 1999, Sun N *et al*., 2008a, 2008b). Building a detailed hypothesis or direct linking of nestin to a particular pathway, however, is more demanding. The Notch signaling pathway is extensively studied and evolutionary conserved signaling mechanism that has been implicated critical in cell fate decisions during development and differentiation in several tissues including muscle (Schuster-Gossler *et al*., 2007, Vasyutina et al., 2007). In myogenic precursor cells the Notch cascade has been described to inhibit the differentiation through the activity of Notch effectors, transcriptional repressors of Hey family, which hamper the expression of MRFs (Buas *et al*., 2010). Avian ortholog of nestin, transitin, has been associated with the Notch signaling in neuronal system as it through a direct interaction regulates the stability and the subcellular localization of Numb, a negative regulator of Notch (Wakamatsu *et al*., 2007). Thus, it would be interesting to analyze the role of nestin in myogenic Notch signaling. The Wnt pathway is another signaling cascade driving the differentiation of myoblasts during regeneration (Brack *et al*., 2008). We have got indications of a possible interaction between nestin and the Wnt effector β-catenin during the myoblast differentiation process which could implicate an involvement of nestin in the Wnt pathway (data not shown). However, further experiments are required to solidly link nestin in this signaling machinery. A specific analysis of nestin interaction partners with the help of immunoprecipitation and mass spectrometry would definitely provide great benefits for nestin studies besides in myoblasts but also in other nestin expressing cell and tissue types.

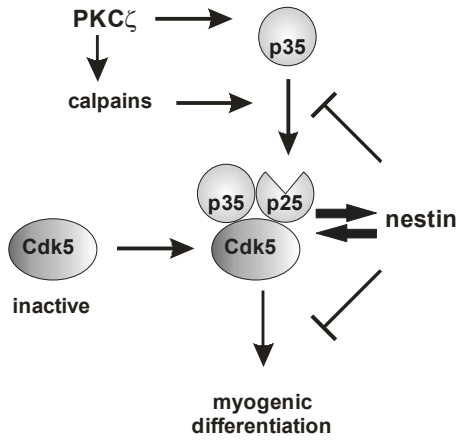

Figure 19. Dynamic interplay between nestin and the PKCξ-Cdk5 signaling axis in differentiating myoblasts. Nestin regulates the progress of myoblast differentiation by controlling the Cdk5/p35/p25 signaling complex through sequestration. The processing of p35 to p25 is critically affected by the nestin scaffold. The interrelationship between nestin and Cdk5 is bidirectional as the PKCξ-Cdk5 signaling cascade regulates the nestin stability and reorganization during myoblast differentiation.

CONCLUDING REMARKS

The last decade of research has broadened the view of IF functions previously considered solely mechanical. The intriguing ability of IFs to modulate the localization and activity of a variety of signaling determinants through scaffolding has proven IFs to be regulators of cell signaling. This feature provides perhaps an explanation for the multimember IF protein family arguing that IFs could function as specialized regulatory elements for distinct cell types and physiological processes. Understanding the role of IFs in signal transduction not only provides insight into the specific IF functions but also places IFs in an interesting position where the modulation of IF expression or structure could be hypothesized as a mechanism to regulate cell signaling.

An earlier work from our laboratory had characterized an interaction between IF protein nestin and the Cdk5/p35 signaling complex. Therefore, in this PhD thesis a strong emphasis was placed on clarifying the functional significance of nestin in the regulation of the Cdk5/p35 complex. Using oxidative stress-induced neuronal apoptosis as a model system we revealed a prominent antiapoptotic function of nestin deriving from the regulation of detrimental Cdk5 activity. Through its ability to anchor the Cdk5/p35 signaling complex, nestin regulated the turnover of p35, the calpain-mediated processing of p35 to a more stable activator, p25, and the subcellular localization of the Cdk5/p35 complex, thereby having a profound influence on Cdk5 activity.

Myogenic development is another physiological process characterized by the concomitant nestin expression and Cdk5 activity. During the work of this thesis we succeeded in elucidating the upstream regulatory mechanisms of Cdk5 in differentiating myoblasts by illustrating PKCξ as a master controller of Cdk5 activity. By means of p35 phosphorylation and calpain activation, PKCξ promoted the calpain-mediated cleavage of p35 to p25, thus enhancing Cdk5 activity. These results expand the understanding of the regulatory mechanisms governing Cdk5 activity. More importantly, our data challenges the current dogma of p25 considered primarily responsible for the uncontrolled and harmful Cdk5 activity during stress and disease, by indicating the utility of p25 when prominent but temporary Cdk5 activation is required. While analyzing the regulation of Cdk5 we made an effort towards understanding the functions of nestin in myogenesis. We observed that the onset and pace of myoblast differentiation were significantly accelerated upon nestin downregulation. The absence of nestin scaffold induced a substantial increase in the generation of p25 indicating that the nestin scaffold sets the pace for myoblast differentiation by affecting Cdk5 activity. Moreover, we detected a dynamic crosstalk between nestin and the PKCξ-Cdk5 signaling axis in differentiating myoblasts. While nestin appeared to determine the Cdk5 activation, nestin reorganization and stability where dependent on the PKCξ-Cdk5 signaling cascade referring to the ability of Cdk5 to modulate its own activity by regulating the stability and assembly-state of the nestin scaffold. Taken together, my PhD thesis provides novel information of the regulation of Cdk5 introducing nestin and PKCξ as key determinants. Forthcoming studies are warranted to define whether this knowledge is of relevance also in the context of CNS development and functionality or neurodegenerative disorders typified by Cdk5 activity.

In this PhD thesis we implemented the analysis of nestin functions in neuronal stress and in myogenesis by using the RNAi technology. We depicted the remarkable ability of

nestin to regulate the progenitor cell fate by affecting critical progenitor cell features, stress tolerance and potential to differentiate, through the scaffolding of the Cdk5/p35 complex. During the course of these studies a nestin KO mouse was generated and is currently under analysis. Detailed dissection of development and tissue regeneration in nestin KO mice not only in terms of the Cdk5/p35 signaling complex but also other cellular factors would greatly benefit the endeavors to characterize the cellular functions of nestin.

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At home in September 2010

Hanna-Mari Pallari

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