To divide or differentiate?

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To my family and friends

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LIST OF ORIGINAL PUBLICATIONS

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I. Pallari H.-M., Lindqvist J., Torvaldson E., Ferraris S.E., He T., Sahlgren C., Eriksson J.E. Nestin as a regulator of Cdk5 in differentiating myoblasts
Mol Biol Cell. 2011 May; 22(9):1539-49

II. Lindqvist J.*, Torvaldson E.*, Gullmets J., Karvonen H., Taimen P., Eriksson J.E. The intermediate filament protein nestin is required for skeletal muscle physiology and regeneration
Manuscript

III. Lindqvist J., Imanishi S.I.*, Torvaldson E.*, Malinen M., Remes M., Örn F., Palvimo J.J., Eriksson J.E. Cyclin-dependent kinase 5 acts as a critical determinant of AKT-dependent proliferation and regulates differential gene expression by the androgen receptor in prostate cancer cells
Mol Biol Cell. 2015 June; 26(11):1971-84

*equal contribution

ABBREVIATIONS

ACh	Acetylcholine
AChR	Acetylcholine receptor
AD	Alzheimer´s disease
ADT	Androgen deprivation therapy
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
AR	Androgen receptor
Bcl-2	B-cell lymphoma 2
CamKII	Ca^{2+}/calmodulin-dependent protein kinase II
Cdk5	Cyclin-dependent kinase 5
Cdk5-SV	Cyclin-dependent kinase 5 splice variant
CHX	Cycloheximide
CNS	Central nervous system
CRPC	Castration resistant prostate cancer
DARPP-32	Dopamine- and cAMP-regulated phosphoprotein, 32 kDa
DMSO	Dimethyl sulfoxide
DN-Cdk5	Dominant negative Cyclin-dependent kinase 5
EDL	Extensor digitorum longus muscle
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FCS	Fetal calf serum
FSH	Follicle-stimulating hormone
GFP	Green fluorescent protein
GLUT-4	Glucose transporter type 4
GR	Glucocorticoid receptor
GSK3β	Glycogen Synthase Kinase 3β
hCG	Human chorionic gonadotropin
ICC	Immunocytochemistry
IDE	Insulin degrading enzyme
IF	Intermediate filament
IFP	Intermediate filament protein
IHC	Immunohistochemistry
IL-6	Interleukin 6
IP	Immunoprecipitation
JNK3	c-Jun N-terminal kinase 3
LAP2α	Lamina-associated polypeptide 2α
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LH	Luteinizing hormone
LLB	Laemmli lysis buffer
L-VDCC	L-type voltage-dependent Ca^{2+} channel
MEF	Mouse embryonic fibroblast
MEF2	Myocyte enhancer factor-2
MEK1	MAP kinase kinase-1
MEK2	ERK activator kinase 2 (MAP kinase kinase 2)
MHC	Myosin heavy chain

MuSK	Muscle specific kinase
MSC	Mesenchymal stem cell
Mst3	Mammalian STE20-like protein kinase 3
mTOR	Mammalian target of Rapamycin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
Nest-640	GFP tagged nestin truncation construct (amino acids 1-640)
NF	Neurofilament
NRG	Neuregulin
NMDAR	N-methyl-D-aspartate receptor
NMJ	Neuromuscular junction
Ndel1	Nuclear distribution protein nudE-like 1
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIKE-A	Isoform A of phosphatidylinositol 3-kinase enhancer
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PKC	Protein kinase C
PPARγ	Peroxisome proliferator-activated receptor gamma
PS	Pseudosubstrate (PKCζ inhibitor)
PSD-95	Postsynaptic density-95
PTEN	Phosphatase and tensin homolog
PTM	Post-translational modification
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
pRb	Retinoblastoma protein
RNAi	RNA interference
SDS-PAGE	Sodium dodecylsulphate- polyacrylamide gel electrophoresis
Sik2	Salt-inducible kinase 2
siRNA	Small interfering RNA
StAR	Steroidogenic acute regulatory protein
STAT3	Signal transducer and activator of transcription 3
T2D	Type II diabetes
TA	Tibialis anterior muscle
TNFα	Tumor necrosis factor α
WAVE 1	Wiskott-Aldrich syndrome protein family member 1
WB	Western blotting

ABSTRACT

The molecular functions of the non-cell cycle-related Cyclin-dependent kinase 5 (Cdk5) have been of primary interest within the neuroscience field, but novel undertakings are constantly emerging for the kinase in tissue homeostasis, as well as in diseases such as diabetes and cancer. Although Cdk5 activation is predominantly regulated by specific non-cyclin activator protein binding, additional mechanisms have proved to orchestrate Cdk5 signaling in cells. For example, the interaction between the intermediate filament protein nestin and Cdk5 has been proposed to determine cellular fate during neuronal apoptosis through nestin-dependent adjustment of the sensitive balance and turnover of Cdk5 activators. While nestin constitutes a crucial regulatory scaffold for appropriate Cdk5 activation in apoptosis, Cdk5 itself phosphorylates nestin with the consequence of filament reorganization in both neuronal progenitors and differentiating muscle cells. Interestingly, the two proteins are often found co-expressed in various tissues and cell types, proposing that nestin-mediated scaffolding of Cdk5 and its activators may be applicable to other tissue systems as well. In the literature, the molecular functions of nestin have remained in the shade, as it is mostly exploited as a marker protein for progenitor cells. In light of these studies, the aim of this thesis was to assess the importance of the nestin scaffold in regulation of Cdk5 actions in cell fate decisions.

This thesis can be subdivided into two major projects: one that studied the nature of the Cdk5-nestin interplay in muscle, and one that assessed their role in prostate cancer. During differentiation of a myoblast cell line, the filament formation properties of nestin was found to be crucial in directing Cdk5 activity, with direct consequences on the process of differentiation. Also the genetic knockout of nestin was found to influence Cdk5 activity, although differentiation per se was not affected. Instead, the genetic ablation of nestin had broad consequences on muscle homeostasis and regeneration. While the nestin-mediated regulation of Cdk5 in muscle was found to act in multiple ways, the connection remained more elusive in cancer models. Cdk5 was, however, established as a significant determinant of prostate cancer proliferation; a behavior uncharacteristic for this differentiation-associated kinase. Through complex and simultaneous regulation of two major prostate cancer pathways, Cdk5 was placed upstream of both Akt kinase and the androgen receptor. Its action on proliferation was nonetheless mainly exerted through the Akt signaling pathway in various cancer models. In summary, this thesis contributed to the knowledge of Cdk5 regulation and functions in two atypical settings; proliferation (in a cancer framework) and muscle differentiation, which is a poorly understood model system in the Cdk5 field. This balance between proliferation and differentiation implemented by Cdk5 is ultimately regulated (where present) by the dynamics of the cytoskeletal nestin scaffold.

INTRODUCTION

The stem cells that derive from the inner cell mass of the blastocyst in the developing embryo are the precursors to all cells and tissue types in the body. Through the process of differentiation, the stem cell loses its totipotency, and acquires instead more specialized functions needed by the particular cell type it will differentiate into. Whereas stem cells have the capacity to divide limitlessly, most differentiated cell types have lost the capacity of cell division in humans, and have entered the quiescent G_0-cell cycle phase. During their lifetime, all tissues are disposed to constant environmental stress, which is harmful for the cells. For example, in response to injury and the consequential loss of cells, tissues need to replenish lost material. This typically occurs through the activation and differentiation of tissue-resident progenitor cells, with the help of an inflammatory response, which aids in the recruitment of appropriate cell types to the site of damage. Thus, most tissues do have the capacity of self-renewal, at least to a certain extent. An example of a constantly renewing tissue is skeletal muscle. Skeletal muscle is prone to injuries of variable extent, including the microdamage that is induced by everyday tasks such as motility and exercise. Exercise-provoked activation of muscle progenitor cells heals the damaged myofibers and contributes to muscle mass increase. The muscle progenitors have limited renewal capacity, and will eventually deplete with time. Muscle resident progenitor cells are also depleted or dysfunctional in disease conditions, such as muscle dystrophies. Tissue damage is strictly regulated by the inflammatory environment, and a correct response that drives the proliferation and differentiation of all cell types involved is necessary for proper healing. A prolonged inflammation can cause stem cell depletion and fibrosis, with dire consequences on tissue function.

An interesting example of flawed differentiation, or rather the lack of differentiation, is cancer. Cancerous cells are generated by accumulation of mutations to the cell´s genome. Eventually, the mutations provide the cancer cells with growth advantage properties that will allow the cells to divide endlessly, defy cell death, resist cell contact-independent growth and acquire a motile phenotype required for metastasis. Cancer cells and stem cells resemble each other in their great proliferation capacity, which in the case of cancer cells, has gone out of reach of the cellular control mechanisms that have evolved to prevent selfish cell behavior. Although limitless proliferation is used to define cancer, malignancies that arise in different tissues have unique molecular characteristics, different origin and are surrounded by a distinctive environment that transforms the cells it surrounds. While mutations in most common oncogenes and tumor suppressors occur in most, if not all cancers, tissue-specific tumor founder mutations reflect the tissue source. In the case of prostate cancer, which is studied in this thesis, the androgen signaling also required for prostate development, plays a key role in prostate carcinogenesis.

As cells are constantly under the strain of various stress factors of variable severity and type that may damage the cells´ proteins and DNA, the cells need a rapid signal transduction system to respond to extracellular cues. All the signals that a cell receives are combined and interpreted by signaling executors inside the cell, allowing an appropriate cellular response to occur. Protein kinases are such crucial components of the cell´s signal transduction system. There are thought to be approximately 500 kinases in humans, which are further subcategorized according to sequence and functional homology (Manning et al., 2002) (Figure 1). These kinases have evolved to mediate highly specialized functions within the cell. They regulate target protein function, localization and activity through addition of negatively charged phosphate groups on serine, threonine or tyrosine residues on target proteins. Thus, phosphorylation, a form of post-translational modification, is a quick and reversible modification that transfers cellular signals within minutes inside the cell, but can also be sustained for extended periods. Protein

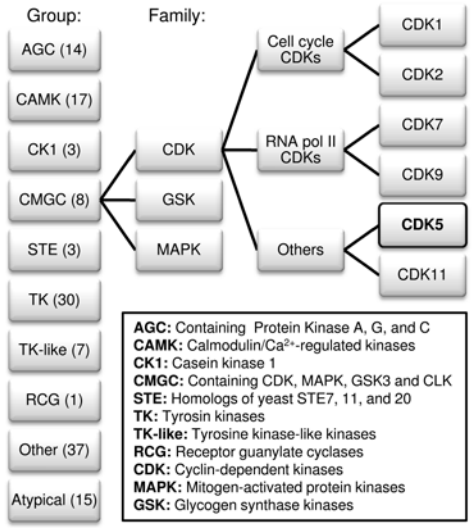

Figure 1 Classification of human kinases. The approximately 500 human kinases are classified into 9 major groups based on sequence comparison. The groups are further sub-categorized into families (the number of kinase families per group is marked in parentheses). In addition, the atypical kinases consist of 8 groups, containing a total of 15 families. Cdk5 belongs to the CMGC group of kinases, which also includes the GSK and MAPK families. Here, the Cdks are classified into three functionally different groups (not homology-based). Modified from Manning et al., 2002.

phosphorylation is actively counteracted by de-phosphorylation by protein phosphatases such as the Ser/Thr phosphatases PP2A and PP1. In addition to phosphorylation, other forms of post-translational modifications (such as ubiquitination, SUMOylation, glycosylation, methylation and acetylation) are thought to be equally powerful in the functional alterations of proteins, and can function as efficient on/off switches for cellular responses.

Protein kinases regulate most signal transduction pathways, and have vast effects on cell division, apoptosis, differentiation, growth, migration and invasion. Deregulation of phosphorylation patterns are acknowledged to contribute to diseases such as neurodegenerative conditions as well as cancer, making protein kinases attractive pharmaceutical targets. Kinase inhibitors are in fact one of the most investigated cancer drug types due to their potential to be very selective, and the fact that many cancer types are often highly dependent on a few signaling pathways, in other words they are "oncogene-addicted". Most oncogenic kinases have broad (pro-proliferative, pro-migratory, anti-apoptotic) and inter-connected functions; therefore, in the case of one-pathway addiction, single kinase inhibitors may have desirable effects in patients. Acquired resistance in response to chemotherapy is, however, common, and leads to adaptation of cancer cells to a new environment. Functional compensation by other oncogenic pathways that were not initially targeted may require further actions and lead to re-design of medication. Thus, to target specific protein kinases for pharmacological purposes, we first need to understand how these kinases work in different contexts, how they are regulated and how they connect with other signaling pathways.

This thesis aimed to understand the roles and regulation of the atypical protein kinase cyclin-dependent kinase 5 (Cdk5) in two model systems: muscle and prostate cancer. On one hand, I have studied how the cytoskeletal protein nestin affects Cdk5 signaling in muscle progenitor cell cultures and in animals lacking nestin. Here I describe that nestin is an essential part of the Cdk5-regulatory axis in muscle differentiation, as it fine-tunes the early stages of the differentiation process and acts as a dynamic scaffold for the kinase. The other project has focused on the role of Cdk5 in prostate cancer proliferation. Although Cdk5 is classically considered to be a key component of neuronal differentiation, recent studies have revealed that Cdk5 plays an essential part in many tissue types during homeostasis and disease. In this work, I depict how this differentiation-related kinase also has pro-proliferative effects on cancer cell growth through its mutual effects on the oncogenic kinase Akt and androgen signaling. Together these studies have assessed some less well understood functions of Cdk5 and have broadened the view of typical Cdk5 functions.

REVIEW OF THE LITERATURE

1 Cyclin-dependent kinase 5 in development and disease

The Cyclin-dependent kinases (Cdks) are a group of more than 20 Ser/Thr protein kinases in humans. They are mainly activated, as their name suggests, through their association with cyclins or cyclin-like proteins. Cdk4, Cdk6, Cdk2 and Cdk1 (Cdc2) are involved in the progression of mammalian cell cycle (G_1 phase; G_1 phase; G_1/S transition, S and G_2 phases; M-phase, respectively) and are activated through the association with their cognate cyclin partners (D-cyclins; D-cyclins; E- and A-cyclins; B-cyclins, respectively). The Cdk family also contains members that are involved in transcription through their interactions with RNA polymerase II (Cdk7, Cdk8, Cdk9 and Cdk11). Unlike the other kinase family members, Cdk5 was originally described as a master regulator of neuronal differentiation and death, but further studies have revealed complex roles for the kinase in several tissue types, with functions in cellular secretion, survival and motility.

1.1 The molecular structure of Cdk5

The 33-kDa Cdk5 was originally categorized in the subfamily of cyclin-dependent kinases according to sequence homology to Cdk1 (~58%) and Cdk2 (~61%) (Hellmich et al., 1992). Formerly known as neuronal cdc2-like kinase (nclk) or PSSALRE, Cdk5 was first cloned from rat brain lysates (Hellmich et al., 1992). In addition, Cdk5 has been identified in most studied animals, including human (Meyerson et al., 1992). Cdk5 is, in fact, highly conserved in mammals (~99%), suggesting an important cellular function for the protein (Tsai et al., 1993). For comparison, *Drosophila* Cdk5 shows 77% sequence homology to mammalian genes (Hellmich et al., 1994). In addition, evidence point towards the existence of a functional Cdk5-homolog in yeast: the Pho85-kinase deletion phenotype can be rescued with Cdk5 expression, at least in terms of restoration of actin cytoskeleton organization and nutrient sensing, which include some of the functions of Pho85 (Huang et al., 1999).

Despite its close structural resemblance to other Cdks, Cdk5 shows unique features that distinguishes it from the other family members. Firstly, all Cdks contain a distinctive amino acid sequence in between the small N-terminal lobe and the α-helical C-terminus, which in the case of Cdk5 is the one letter amino acid sequence PSSALRE (Meyerson et al., 1992); hence its original name. In between the N- and C-termini of all Cdks lies an adenosine triphosphate (ATP)-directing activation loop that also contributes to substrate specificity. In contrast to other Cdk family members, Cdk5 can associate with certain cyclins, but is mainly activated by non-cyclin proteins. The third major factor that distinguishes Cdk5 from cell cycle Cdks is its independence of activation loop phosphorylation upon activation, a crucial step in classical Cdk activation. Instead, activator binding promotes an active conformation of Cdk5, while simultaneously affecting kinase substrate specificity (Qi et al., 1995; Tarricone et al., 2001). Classically, c-Abl (Zukerberg et al., 2000) and Fyn kinase (Sasaki et al., 2002)-mediated phosphorylation of Cdk5 Tyr15 is thought to promote kinase activity in neurons, while the phosphorylation of the corresponding residues on classical Cdks has a strong inhibitory effect on kinase activity. On the other hand, recent data suggests that Fyn increases Cdk5 activity by promoting p35 stability, and that Tyr15-phosphorylated Cdk5 could in fact reside in an inactive monomeric state (Kobayashi et al., 2014). Thus, phospho-antibodies directed at Cdk5 Tyr15 are not a suitable tool for assessing the Cdk5 activation state. In addition, Cdk5 activity has been suggested to be post-translationally repressed by S-nitrosylation of Cys83 in the ATP binding

pocket, a motif not present in other Cdks (Zhang et al., 2010b). While these structural features differentiate Cdk5 from its family members, the substrate consensus phosphorylation sequence of these kinases is strikingly similar. Cdk5 phosphorylates serines (Ser) and threonines (Thr) followed by a proline (Pro) on substrates that contain the consensus sequence (Ser/Thr)ProX(Lys/His/Arg), where X is any amino acid that is followed by one of the basic amino acids lysine, histidine or arginine (Songyang et al., 1996). Thus, Cdk5 and Cdk1 do share several substrates.

In 2010, two independent reports indicated the presence of a ubiquitously expressed novel Cdk5 splice variant (Cdk5-SV) (Kim et al. 2010; Li et al. 2010). The truncated Cdk5-SV has a molecular weight of 29 kDa, and shows a prominent nuclear/perinuclear localization (Kim et al. 2010; Li et al. 2010). Interestingly, Kim and co-authors (2010) reported that exogenous Cdk5-SV co-localized with centrosomes, which according to the authors, possibly signified a cell division-related role. It should be highlighted in this context that Cdk5-SV is detected by most commercial antibodies (including those described in this thesis). Thus, it is impossible to distinguish between the functions of the distinct splice variants from previous literature, as most studies are investigating endogenous Cdk5. However, there is currently no evidence of Cdk5-SV kinase activity in tissues.

1.2 Mechanisms of Cdk5 activation

Cdk5 activation unconditionally requires binding of activator proteins. The certainly most studied activation mechanism of Cdk5 is through its interaction with p35, as well as its cleavage product p25, which is generated by calpain-dependent cleavage of the mother protein. The Cdk5 interaction with p35 occurs between amino acids 138 and 291 on p35 (Amin et al., 2002; Poon et al., 1997). Recent studies have expanded on the mechanisms that regulate Cdk5 activation also beyond classical neuronal models and challenged the previous view of the activation process.

1.2.1 p35 and its proteolytic cleavage products

Cdk5 and p35 (as well as its proteolytic cleavage product p25) were first discovered by pull-down of the active complexes from brain lysates (Lew et al., 1992a; Lew et al., 1994; Tsai et al., 1994). p35 (also known as CDK5R1, and previously nck5a) is unrelated to the cyclin protein family; yet the crystal structure of p25 reveals that the three dimensional conformation shows striking similarities to cyclin A, as the activator contains a cyclin box fold-like motif in the Cdk5-binding region (Tarricone et al., 2001). Hence the tertiary structure of the holokinase allows comparable interactions between the subunits similar to classical cyclin-Cdk pairs; an interaction, which advances ATP-binding and substrate recognition. Activator binding to Cdk5 occurs through interactions with the kinase activation loop and the Cdk5-specific PSSALRE-region (Tarricone et al., 2001).

Cleavage of p35 to p25 by calpains has broad consequences on Cdk5 activity through effects on both the stability and localization of the active kinase complex. p35 is normally associated with the plasma membrane, but can also be detected in perinuclear areas in the cytoplasm. If the p35 myristoylation site Gly2 is mutated, the intracellular localization of p35 is dramatically altered, shifting towards a diffuse cytoplasmic and partially nuclear pool (Asada et al., 2008). As calpain cleavage removes the myristoylated p35 N-terminus, the direct outcome of calpain activity is generation of freely motile kinase-p25 complexes, which tend to accumulate in the nucleus (Figure 2). The myristoylation of the N-terminal Gly2 on p35 is adequate for membrane recruitment, but positively charged Lys residues further stabilize the membrane association (Asada et al., 2008). The N-terminal Lys cluster has dual functionality, as the cluster also

interacts with importins to facilitate nuclear import of the protein (Fu et al., 2006). Therefore, the classical view of p35 activator localization has been challenged. Nuclear p35 is known to be important for Cdk5-mediated neuronal cell cycle suppression also in the nucleus (Zhang et al., 2010a), supporting the notion that its nuclear localization may have a true biological function.

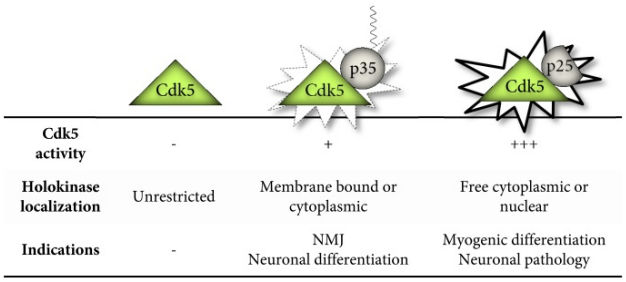

Figure 2 Cdk5 activity and localization is regulated by activator binding. The processing of p35 affects both the activation of Cdk5 as well as its localization. Both p35 and p25 do have physiological functions, but detrimental and disease-related p25 generation is an important mechanism in neurodegenerative disease. NMJ: neuromuscular junction.

The second important distinction between p35 and p25 is the difference in their half-life. p35 is rapidly degraded by the proteasome and has a half-life of 20-30 minutes, while p25 shows a notable increase (2-3 times) in protein stability (Patrick et al., 1998a). It is believed that the p10 fraction of p35 contains degradation elements that promote both proteasomal degradation and calpain cleavage at the membrane (Minegishi et al., 2010). The first evidence of calpain-mediated cleavage of p35 came from evidence of neurotoxic (ischemia, amyloid β-peptide and hydrogen peroxide-induced) insults in mice and cultured neurons (Kusakawa et al., 2000; Lee et al., 2000), and the results paved the way for a whole era of intensive Cdk5 research within the area of neurodegenerative diseases.

Calpains are a family of calcium-dependent cysteine proteases that are crucial executors of signaling processes including apoptosis, cell motility and differentiation, through the modification of cytoskeletal structures and signaling proteins, and the calpains are thought to play a role in many pathological events, such as neurotoxicity (reviewed in Goll et al. 2003; Sorimachi & Ono 2012; Sorimachi et al. 2010). Calpains are also important for maintenance of muscle physiology, and calpain mutations have been detected in muscle wasting patients (reviewed in Beckmann & Spencer 2008; Kramerova et al. 2007). Calpains differ in their functionality from the other protein degrading machineries in the cell (e.g. the ubiquitin-proteasome system) in that they are retained inactive in cells, and are quickly activated upon calcium binding, without the need of additional components. Interestingly, it is thought that calpain substrates are recognized based on the spatial structure rather than primary consensus sequence. Both of the ubiquitously expressed classical calpains, μ-calpain (calpain 1) and m-calpain (calpain 2), cleave p35 (Lee et al., 2000).

In a complex auto-regulatory loop, Cdk5 itself regulates p35 processing through phosphorylation. By dual actions, the Cdk5-mediated p35 phosphorylation (Ser8 and/or Thr138) makes the activator more prone to ubiquitination and proteasomal degradation, while simultaneously decreasing its susceptibility to calpain cleavage in order to suppress p25 generation (Kamei et al., 2007; Patrick et al., 1998b; Saito et al., 2003). In this way, Cdk5 can restrict its own activity to prevent unfavorable kinase activity. In addition to Cdk5 itself, two protein kinase C (PKC) isoforms, namely δ (Zhao et al., 2009) and ζ (de Thonel et al., 2010), are known to phosphorylate p35 and regulate its turnover. Whereas PKCζ phosphorylation of Ser33 promotes p25 generation in a myogenic context (de Thonel et al., 2010), PKCδ appears to phosphorylate p35 on serines 59, 65 and/or 124 (a specific site has not been determined), having a stabilizing effect on the activator in neurons (Zhao et al., 2009). In this fashion, PKCδ acts upstream of Cdk5 during the developmental radial migration of cortical neurons. Ser91 in turn is phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II (CamKII) in neurons

(Hosokawa et al., 2010) and salt-inducible kinase 2 (Sik2) in pancreatic β-cells (Sakamaki et al., 2014), which negatively regulates p35 stability in the latter case. The phosphorylation sites are highlighted in Figure 3.

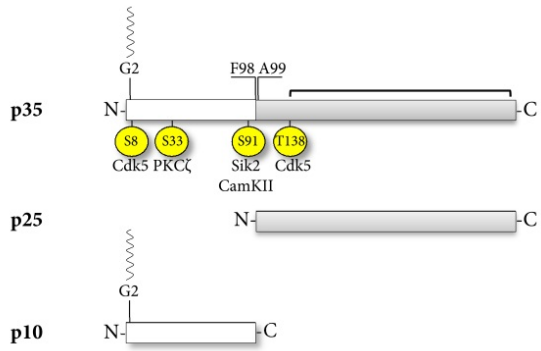

Figure 3 The processing of p35 to p25. Calpains cleave p35 to create two fragments; p25 and p10. Due to loss of the myristoylated Gly2, p25 is freely motile within the cells. Known phosphorylation sites of p35 are marked in yellow. The Cdk5 interaction site on p35 is highlighted with a bracket.

The expression of p35 is under the control of an array of extracellular signals (e.g. through laminin-induced integrin activation) and growth factor stimulation (such as nerve growth factor) (Harada et al., 2001; Li et al., 2000). The transcription factor Early growth response 1 (Egr1) has in neuronal PC12 cells been shown to induce p35 mRNA expression in response to nerve growth factor or tumor necrosis factor α (TNFα) (Harada et al., 2001; Utreras et al., 2009). Also, the transcription factor heat shock factor 2 directly binds to the p35 gene promoter and is essential for proper p35 expression and Cdk5 activity in cortical neurons (Chang et al., 2006). Thus, the cell can accommodate its p35 levels and Cdk5 activity needs in response to environmental changes through a multitude of pathways, which are highly dependent on the cellular environment.

In contrast to $Cdk5^{-/-}$ mice that die perinatally (Ohshima et al., 1996), the deletion of p35 in mice causes less dramatic phenotypes. $p35^{-/-}$ mice are viable and show no gross body changes, but they do have defects in cortical lamination due to abnormal neuronal migration, as well as structural changes in some other areas of the brain (Chae et al., 1997). The p35 null mice do show a compensatory increase in the expression of the Cdk5 activator p39 in brain (Takahashi et al., 2003), which is likely to alleviate certain phenotypic characteristics. Compared to WT mice, the p35 knockout mice showed increased adult lethality probably caused by spontaneous seizures (Chae et al., 1997). Other organs, such as skeletal muscle, have not been studied in detail. Outside the central nervous system (CNS), p35 is expressed in the developing myotome from embryonic day 12 (E12) (Zheng et al., 1998), among many other tissues. It should, however, be noted that Cdk5 activator levels (mRNA or protein) are generally relatively low in most other tissues compared to brain lysates (Lew et al., 1994; Tsai et al., 1994). Therefore, the role of p35 in other tissues was long overlooked, as it was challenging to detect its expression, especially at a basal level. In many tissues, a specific stimulus or stress is often required for up-regulation of Cdk5 activators and Cdk5 activity.

1.2.2 Cdk5 activation by p39

Studies on the Cdk5 activator p39 (CDK5R2) have been hampered by the lack of well-functioning antibodies, and most likely the early found strong disease association of p35/p25, as well as the accompanying interest in that specific research area. Sequence alignment of p39 and p35 reveals 57% amino acid similarity between the proteins (Tang et al., 1995). The two proteins are processed analogously by calpains (Patzke and Tsai, 2002b). The calpain cleaved fragment of p39 is correspondingly termed p29, and is like p25, not targeted to the membrane due to loss of its N-terminal myristoylated residue upon proteolytic cleavage (Asada et al., 2008). The biological role and regulation of calpain-dependent cleavage of p29 is not well understood. Compared to p35, the half-life of p39 is significantly longer (Minegishi et al., 2010),

but the contrary, the Cdk5/p39 interaction is weak compared to the Cdk5/p35 complex (Saito et al., 2013). Interestingly, the in vitro substrate specificity of Cdk5/p39 is also different (preferentially tau) from that of Cdk5/p35 (preferentially histone H1) (Takahashi et al., 2003), but it is not clear if p35 and p39 have separate functions in vivo.

Whereas disruption of the p39 gene does not cause apparent phenotypes, the $p35^{-/-}/p39^{-/-}$ double knockout mice (Ko et al., 2001) are identical to mice lacking Cdk5 (Ohshima et al., 1996), demonstrating the need of either p35 or p39 in regulation of Cdk5 activity during neuronal differentiation. As restoration of one allele of p35 to the double-knockout restores partial neuronal functionality, while one allele of p39 does not, p35 is believed to be the major Cdk5 activator in neuronal models (Ko et al., 2001). Both p35 and p39 are expressed widely in the CNS during embryonic development, but some spatial and temporal differences can be detected between the two proteins, such as the prevalent p39 expression in the spinal cord during embryogenesis (Zheng et al., 1998) and the postnatal increase in p39 mRNA in the cerebral cortex (Takahashi et al., 2003). Cdk5/p39 interaction and activity has been described in neurons and the complex may functionally be coupled to growth cone maintenance (Humbert et al., 2000). Cdk5/p39 also phosphorylates the cytoskeletal protein tau during brain development (Takahashi et al., 2003). Phosphorylated tau shows less affinity towards microtubules (possibly reducing microtubule stability), and the complex may therefore be involved in the developmental regulation of microtubule dynamics. Cdk5/p39 also regulates lamellipodial actin dynamics through Rac1 (Ito et al., 2014). In addition, Cdk5/p39 is implicated in oligodendroglial (the myelinating cell of CNS) differentiation (Bankston et al., 2013). In contrast to p35, p39 mRNA expression has not been reported in muscle tissue, but the protein has been detected with antibody recognition in rat primary myotubes (Johansson et al., 2005). Both activators have also been described at mRNA and protein levels in pancreatic β-cells (Lilja et al., 2001).

1.2.3 Cdk5 and cyclin partners

Podocytes are specialized epithelial cells that surround the vasculature of glomeruli in the kidney. Their characteristic highly branched appearance is reflective of their function; by wrapping their foot protrusions around blood vessels, the cells selectively filter blood and constitute a crucial part of the kidney´s homeostatic machinery. The expression of Cdk5/p35 correlates with the differentiated state of podocytes and both proteins can be distinguished at the edges of the podocyte protrusions (Griffin et al., 2004). Cdk5 is thought to have two main functions in podocyte biology. As the inhibition or downregulation of Cdk5 causes a collapse of podocyte protrusions, possibly as a consequence of actin filament reorganization, its presence at the cell periphery may be required for maintenance of podocyte morphology (Griffin et al., 2004). Intriguingly, active Cdk5 also acts as a pro-survival factor after podocyte injury in vivo, a process which is controlled not only by the classical activator p35, but also by the atypical cyclin I (Brinkkoetter et al., 2009). Cyclin I is the only identified cyclin partner with the ability to activate Cdk5 (Brinkkoetter et al., 2009; Brinkkoetter et al., 2010). Although both activators seem to be present in podocytes, the Cdk5/p35 and Cdk5/cyclin I complexes differ in their functionality. The substrate specificity of Cdk5 in vitro is shifted towards tau rather than histone H1 when complexed with cyclin I (Brinkkoetter et al., 2009). This may also explain the differences in the involved cell survival signaling pathways in p35 and cyclin I null mice, even though both conditions sensitize podocytes to injury in a glomerulonephritis-disease model (Brinkkoetter et al., 2009; Taniguchi et al., 2012). While p35 null podocytes have less anti-apoptotic Bcl-2, cyclin I null cells show suppressed Extracellular signal-regulated kinase (ERK)-signaling and decreased levels of anti-apoptotic Bcl-XL (Brinkkoetter et al., 2009; Brinkkoetter

et al., 2010; Taniguchi et al., 2012). Whether the structural and survival-related roles of Cdk5 in podocytes are linked, needs to be clarified.

Another independent study regarding the post-translational regulation of cyclin I during cell cycle was reported by Nagano and colleagues (2013). They showed that cyclin I protein levels are regulated by ubiquitin-proteasome-mediated degradation during G_2-M phases of the cell cycle, and that its presence is required for normal HeLa cell proliferation (Nagano et al., 2013). Curiously, the protein levels of Cdk5 in synchronized HeLa cells oscillated with cyclin I levels, suggesting that Cdk5 may, after all, be actively regulated during cell cycle, at least in cancer cells. The function and activity of Cdk5 during HeLa cell cycle, and the nature of the Cdk5/cyclin I complex remain elusive.

The fact that Cdk5 activation can occur through association with a novel cyclin partner does evoke many questions, and may simultaneously expose exciting new prospects in the research field. As demonstrated by Brinkkoetter and co-authors (2009), the binding of atypical activator partners may shift the substrate specificity of the Cdk5 kinase. This observation defies the classical Cdk5 activity assay, where Cdk5 activity is measured by histone H1 phosphorylation. Thus, the fact that Cdk5 activity has not been detected in most tissues or cell lines (Tsai et al., 1993) should be interpreted with caution. Furthermore, it was demonstrated that Cdk5/p35 and Cdk5/cyclin I complexes can co-exist in cells, and they may simultaneously regulate survival signaling pathways at different levels. Thus, the generality of the actions of the Cdk5/cyclin I complex should be revisited, as cyclin I is present in most tissues, at least at mRNA level (Jensen et al., 2000).

In addition to cyclin I, also E- and D-type cyclins are known to bind to Cdk5. Nevertheless, Cyclin E seems to act as a suppressor of Cdk5 activity, perhaps through inhibition of Cdk5/p35-complex formation by competition (Odajima et al., 2011). The Cdk5/cyclin E complex lacks kinase activity towards histone H1, Retinoblastoma protein (pRb) and synapsin 1, all of which are potent Cdk5 substrates. As a consequence of cyclin E knockout, Cdk5 activity was boosted, causing a reduction of dendritic spine and synapse number in vivo (Odajima et al., 2011). Cyclin D has been co-immunoprecipitated with Cdk5, but the complex has no activity measured by histone H1 phosphorylation (Guidato et al., 1998; Zhang et al., 1993); rather, cyclin D may function, similarly to the E-isoform, as a p35 competitor.

Several research papers collectively infer that Cdk5, in fact, does regulate the cell cycle, although in a somewhat indirect fashion, and does therefore in many aspects behave similarly to its protein family members. Overexpression studies of WT and inactive Cdk5 in HeLa cells revealed that Cdk5 can localize to the midbody during cytokinesis (Lee et al., 2010). Intriguingly, $Cdk5^{-/-}$ mouse embryonic fibroblasts (MEFs) show increased occurrence of aneuploidy, possibly suggesting that the presence of Cdk5 may be important for proper cell cycle progression (Lee et al., 2010). $p35^{-/-}/p39^{-/-}$ mice are also smaller in body size, pointing to a growth defect in affected mice (Ko et al., 2001). Thus, in certain contexts, Cdk5 may act to substitute at least some classical Cdk functions in more differentiated cell types.

1.3 Developmental and physiological functions of Cdk5

The actions of Cdk5 have mainly been studied in neuronal models, where a wide range of Cdk5 substrates have been characterized. Cdk5 mRNA and protein are expressed most abundantly in the brain, but can in fact be detected in most tissues (including heart, skeletal muscle, kidney and pancreas) and in many cell lines at varying levels (Meyerson et al., 1992; Tsai et al., 1993). Yet Cdk5 activity, as measured by immunoprecipitation and subsequent phosphorylation of histone H1, is relatively limited in many tissues and cells compared to neurons (Tsai et al., 1993). As mentioned previously, Cdk5 activity may not in all cases be directed towards histone,

and therefore, the lack of Cdk5 activity by histone H1 kinase assay does not exclude a function for the kinase. In addition, Cdk5 functions have often been revealed only after stress situations, whereas it may have no evident role under normal circumstances. Many surprising new functions for Cdk5 have been proposed in recent years. The classical functions of Cdk5 in neurons will not be the prime focus of this overview and are only briefly summarized in the following section. Instead, I will highlight some of its more atypical and less understood functions in mammalian physiology.

1.3.1 Cdk5 - a major regulator of neuronal development and function

The first evidence for a role for Cdk5 in neuronal function came from primary neuronal cultures, when expression of dominant negative Cdk5 (Asp145→Asn or Lys34→Thr) was found to robustly decrease dendrite length of cultured rat neurons (Nikolic et al., 1996). Genetic targeting of Cdk5 confirmed its role in neuronal development: most $Cdk5^{-/-}$ mice show embryonic lethality at E18.5, and the few mice that survive until birth die within the first 12 hours (Ohshima et al., 1996). The principal cause for the high prenatal death rate is the severe cortical layering defect caused by the absence of Cdk5 signaling (Ohshima et al., 1996). Also Cdk5 or p35 disruption in the fruit fly causes axon patterning complications and locomotion problems (Connell-Crowley et al., 2000; Connell-Crowley et al., 2007; Kissler et al., 2009). To date, a large number of Cdk5 substrates have been identified in neurons, and together they have uncovered that Cdk5 indeed acts at several levels of neurogenesis (Table 1).

Table 1 Summary of neuronal Cdk5 substrates.

	Selected substrates	Implications	References
Migration and cytoarchitecture	FAK, doublecortin, Ndel1, Dixdc1	Microtubule dynamics and neuronal migration	(Niethammer et al., 2000; Singh et al., 2010; Tanaka et al., 2004a; Xie et al., 2003)
	Mst3, p27Kip1	RhoA-mediated actin reorganization and motility	(Tang et al., 2014; Kawauchi, Chihama, Nabeshima, & Hoshino, 2006)
	Neurofilaments	Intermediate filament organization	(Pant et al., 1997; Sun et al., 1996)
	β-catenin	N-cadherin-dependent cell adhesion	(Kesavapany et al., 2001; Kwon et al., 2000)
Synapse signaling	PSD-95, NMDAR, DARPP-32	Regulation of pre- and postsynaptic dopaminergic and glutaminergic neurotransmission	(Chergui et al., 2004; Li et al., 2001; Morabito et al., 2004)
	Munc-18/dynamin 1, amphiphysin 1	Exocytosis/endocytosis at the synapse	(Fletcher, 1999; Tan et al., 2003; Tomizawa et al., 2003)
	Ephexin 1, WAVE 1	Synaptic plasticity and spine formation	(Fu et al., 2007; Kim et al., 2006)
	Synapsin 1	Presynaptic vesicle number and trafficking	(Verstegen et al., 2014)
Death and survival	p53, ATM, pRb, MEF2	Positive regulation of apoptosis during neurotoxicity	(Zhang, Krishnamurthy, & Johnson, 2002; Tian, Yang, & Mao, 2009; Hamdane et al., 2005; Gong et al., 2003)
	JNK3, Bcl-2	Advancing neuronal survival	(Li et al., 2002; Cheung, Gong, & Ip, 2008)
	p27Kip1, E2F1	Neuronal cell cycle suppression and cytoprotection (kinase activity independent)	(Zheng et al., 2010a)

Cdk5 is acknowledged to exert its kinase activity towards a large repertoire of cytoskeletal components in neurons, and thereby the kinase effectively molds neuronal architecture and regulates neuronal differentiation. Through phosphorylation of multiple substrates linked to the microtubule cytoskeleton, Cdk5 is believed to directly modulate the migratory capability of cortical neurons, with the consequence of the severely disrupted cortical architecture that was originally observed in the Cdk5 null mouse (Ohshima et al., 1996). In addition, Cdk5 has actin-linked substrates that affect motility of neurons, but more prominently, its actin-regulating substrates are implicated in remodeling of neuronal processes and the maintenance of synaptic structures. At the synapse, the Cdk5-mediated phosphorylation of the secretory machinery and the neurotransmitter pathway is essential for proper neuronal communication, and in doing so, the kinase directly affects the processes of learning, memory formation and addiction (Table 1 and references therein). Recently, conditional knockout of Cdk5 in the oligodendrocyte lineage has revealed a role for Cdk5 in oligodendrocyte maturation and axonal myelination during development and injury through regulation of the Akt-mammalian target of Rapamycin (mTOR) signaling pathway (Luo et al., 2014; Yang et al., 2013). In contrast to neurons, Cdk5-dependent myelination seems to be driven by p39 rather than p35 (Bankston et al., 2013).

Hyperactivation and re-localization of Cdk5 causes hyperphosphorylation of several of the targets described in Table 1, which has dramatic consequences on ischemia- and stroke-triggered brain injury, and the accompanied neuronal loss in the pathology of neurodegenerative diseases (e.g. Alzheimer´s disease, Parkinson´s disease, amyotrophic lateral sclerosis). In fact, most of these processes involve p25 generation. Ever since the Cdk5/p25 complex was connected to the pathological hallmarks of Alzheimer´s disease and neuronal death (Patrick et al., 1999), there has been an active search for mechanisms that underlie the observed pathology. Enormous efforts have been put into the understanding of the apparently dual roles of Cdk5 in neuronal apoptosis, and the kinase is today portrayed as both a major executor of neurotoxic apoptosis, as well as a neuroprotective kinase under specific conditions. Cdk5 activator cleavage and degradation that change kinase activity and intracellular localization, are under strict regulation within the cell, and are essential in determination of the anti- and pro-apoptotic functions of Cdk5 (Lee et al., 2000; Patrick et al., 1999). Not only is Cdk5 an indispensable component of the neuronal differentiation and death signaling machinery, but several research papers have highlighted a role in pain signaling, where Cdk5 activity is regulated by inflammation in peripheral neurons (Pareek et al., 2006). Depletion of Cdk5 activity causes a delayed nociceptive heat response in p35 null transgenic mice (Pareek et al., 2006). Several pain-linked Cdk5 substrates have been identified in peripheral nerves that contribute to nociceptive signaling, including the protein kinesin-3 family member 13B that transports synaptic cargo (Xing et al., 2012) and the calcium channel transient receptor potential vanilloid 1 (Pareek et al., 2007).

1.3.2 Cdk5 in muscle development and differentiation

Given the dramatic brain phenotype of the Cdk5 knockout mouse and the fact that the mouse is perinatally lethal, the function of Cdk5 in non-CNS tissues has remained in the shade. As follows, the information on Cdk5 molecular functions in muscle is restricted. A few key research articles contributed to early findings that did however place Cdk5 as an important player in muscle differentiation and development. Lazaro and colleagues (1997) first demonstrated that Cdk5 is indeed expressed and active during serum withdrawal-induced differentiation of murine C2C12 and rat L6 myoblasts, and that expression of a dominant negative (DN-Cdk5) in myoblasts inhibited differentiation. Cdk5 activity peaks at circa 48 hours after initiation of differentiation, when myotubes are readily forming and actively fusing (Lazaro et al., 1997). In Philpott et al. (1997), a different approach was utilized; they took advantage of microinjection of DN-Cdk5 to *Xenopus* embryos with the consequence of

disruption of somite development and patterning in the animals. This effect could be rescued with simultaneous expression of WT plasmid. Intriguingly, DN-Cdk5 expression robustly abrogated the expression of the myogenic factor MRF-4, as well as MyoD (albeit the latter to a lesser extent), without any obvious effect on Myf-5, suggesting that Cdk5 acts also at the level of myoblast commitment. However, Cdk5 does not seem to phosphorylate MyoD in vitro (Kitzmann et al., 1999). While Cdk5 activity peaks at 48 hours of differentiation in C2C12 myoblasts, it is possible that the kinase has multiple functions and substrates during differentiation, explaining the observed effect on both early commitment as well as later differentiation. Interestingly, superfluous Cdk5 activity established through p35 expression also seems to hamper proper muscle development (Philpott et al., 1997), suggesting that proper regulation of Cdk5 activity is crucial for myogenesis. In addition, one report demonstrates that Cdk5 or p25 overexpression negatively affects an activatory auto-phosphorylation site of CamKII in C2C12 myoblasts, an observation that may predominantly be relevant for neuromuscular junction signaling rather than differentiation per se (Zhu et al., 2013).

The PKC superfamily consists of a large group of Ser/Thr kinases with functions in most aspects of cell biology, including proliferation, differentiation, migration as well as in the processes of inflammation and cancer (Hirai, 2003; Rosse et al., 2010). PKCs are further subcategorized according to their activation mechanism into typical and novel PKCs that are activated by diacylglycerol binding, and atypical PKCs (including PKC ζ), which are diacylglycerol-independent. The activity of the atypical PKCζ isoform, is required for myoblast differentiation (de Thonel et al., 2010), where it controls the process of differentiation through phosphorylation-mediated regulation of p35 turnover (de Thonel et al., 2010). During myoblast differentiation, PKCζ is required for Cdk5 activation by dual mechanisms: firstly, PKCζ directly phosphorylates Ser33 of p35, thereby increasing its cleavage to p25. The phosphodeficient S33A p35 mutant is indeed more resistant to calpain cleavage. Secondly, PKCζ phosphorylates and affects the activity of calpains 1 and 3. Inhibition of calpain activity, which obstructs p25 cleavage, hampers myogenic differentiation (de Thonel et al., 2010). Therefore, PKCζ is a major upstream regulator of Cdk5 activity and muscle cell differentiation.

Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) phosphorylates plasma membrane phosphatidylinositols to create the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3), to which pleckstrin homology domain containing proteins (such as Akt) can anchor to. Through activation of Akt and other downstream effectors, the PI3K pathway regulates a plethora of signaling events affecting cell proliferation, survival, motility, metabolism and differentiation (reviwed in Brazil & Hemmings 2001; Manning et al. 2002). In muscle, Akt activity is important especially for muscle fiber anabolic growth (Blaauw et al., 2009; Bodine et al., 2001) and glucose metabolism (Bouzakri et al., 2006; Cleasby et al., 2007), but the kinase is also involved in muscle differentiation (Gardner et al., 2012; Héron-Milhavet et al., 2006; Rotwein and Wilson, 2009). The inhibition of Akt kinase activity with the PI3K inhibitor LY294002 during L6 rat myoblast differentiation was, interestingly, found to disturb Cdk5 activity (Sarker and Lee, 2004). Although a detailed mechanism for the observed phenomenon has not been described, the work indicates that PI3K-Akt signaling may directly or indirectly be involved in Cdk5 activation. Akt activity is increased rapidly upon induction of differentiation (within 24 hours) (Sarker and Lee, 2004; Tamir and Bengal, 2000), placing it temporally upstream of the reported maximal Cdk5 activation. As the PI3K pathway positively affects PKCζ activity in other model systems (Mendez et al., 1997; Neri et al., 1999; Standaert et al., 1997), it is tempting to speculate that they all would lay within the same signaling network also in myocytes.

Up to the present time, the only described association partners of Cdk5 in differentiating myoblasts are the sarcomere-associated protein Bin1 (Fernando et al., 2009) and the

cytoskeletal intermediate filament protein nestin (Sahlgren et al., 2003) (described further in: 2.3.2 Interplay between Cdk5 and nestin in muscle models). Although Cdk5 can be immunoprecipitated with Bin1 from differentiating myoblasts, there is no further data on the details of the interaction. Bin1 seems to be crucial for sarcomere assembly, and the authors propose a possible titin-Bin1-Cdk5 interaction that nevertheless remains unconfirmed (Fernando et al., 2009). Titin is a sarcomeric protein, which is heavily phosphorylated during myoblast differentiation on Cdk consensus sites (Gautel et al., 1993), but Cdk5-dependent phosphorylation has never been shown. Surprisingly, there are no other published studies to date addressing the actual targets of Cdk5 in differentiating myoblasts that would rationalize the observed muscle phenotype in various models, leaving a significant area of Cdk5 research unrecognized.

1.3.3 Differentiation of neuromuscular junctions requires Cdk5 activity

Skeletal muscle is innervated by motor axons at specialized intersections, termed neuromuscular junctions (NMJs). NMJs are not only important for muscle contraction, but are essential for the active maintenance of muscle mass throughout the lifespan of the organism. The postsynaptic (muscle) side of the NMJ consists of a specialized cluster of signaling proteins that transduce signals from the synaptic cleft to the muscle. Acetylcholine receptors (AChR), which bind the neurotransmitter acetylcholine (ACh), are dynamically regulated by positive and negative signals that cluster or disperse the transmembrane proteins during NMJ formation. Through binding to its cognate ErbB receptors and consequent activation of ERK-signaling, the neuronally secreted Neuregulin-1 (NRG) promotes transcription of postsynaptic genes, including AChR. The proteoglycan agrin is one of the central molecules that positively regulate AChR clustering through interactions with the muscle specific kinase (MuSK) receptor. MuSK-initiated signaling maintains the AChR clusters on the post-synaptic side of the junction. The active regulation of the NMJ during development allows a flexible spatiotemporal innervation pattern of muscle where it is most required.

The expression of Cdk5 in muscle is concentrated most obviously at NMJs, and both Cdk5 and p35 are regulated by NRG-induced signaling (Fu et al., 2001). Cdk5 was shown to phosphorylate and activate ErbB2/3 receptor tyrosine kinase signaling in response to NRG treatment, thus positively regulating AChR transcription both in vitro and in vivo (Fu et al., 2001; Fu et al., 2005). Genetic ablation of Cdk5 causes an increase of embryonic NMJ width and dispersion in both diaphragm and skeletal muscle, consequently impeding motor skills of the few newborn pups that survive until birth (Fu et al., 2005). In addition, Cdk5 directly phosphorylates the transcription factor signal transducer and activator of transcription 3 (STAT3), and promotes its activity after NRG stimulation of C2C12 myotubes (Fu et al., 2004). Through these mechanisms, Cdk5 is involved in the positive regulation of NMJ development.

On the other hand, also ACh, which acts as to disperse AChR clusters, induces Cdk5 activity (Lin et al., 2005). Thus, acting in an opposing manner, Cdk5 activity also increases AChR dispersion, unless the receptors are stabilized by specific cluster-promoting signals (Lin et al., 2005). The exact mechanism has not been identified yet, but is unlikely to involve direct AChR phosphorylation by Cdk5. Thus, the Cdk5-mediated AChR clustering occurs in conjunction with agrin signaling; if the AChR clusters are on the other hand not stabilized by agrin, Cdk5 acts instead negatively on the AChR clusters in an ACh-dependent manner (Figure 4).

Moreover, Cdk5 knockout mice also show Schwann cells defects (Fu et al., 2005), and genetic disruption of Cdk5, or DN-Cdk5 expression in the muscle, causes increased presynaptic neurotransmitter release, and more frequent generation of postsynaptic potentials (Fu et al., 2005; Zhu et al., 2013). These observations highlight that Cdk5 is required for NMJ

maintenance at both the pre- and postsynaptic sides of the junction. In addition, postsynaptic Cdk5 activity is involved in regulation of presynaptic events, perhaps through regulation of retrograde nitrogen oxide signaling (Zhu et al., 2013). Cdk5/p35 also phosphorylates and binds to the Cdk-related kinase Pctaire-1, which is localized to NMJs, but the biological relevance of the interaction is not comprehended (Cheng et al., 2002). Although Cdk5 kinase activity is required for proper NMJ development, the NMJ signaling pathway that is regulated by the kinase is likely to be distinct from the one involved in myogenic differentiation, which involves NMJ-independent myoblast determination and differentiation.

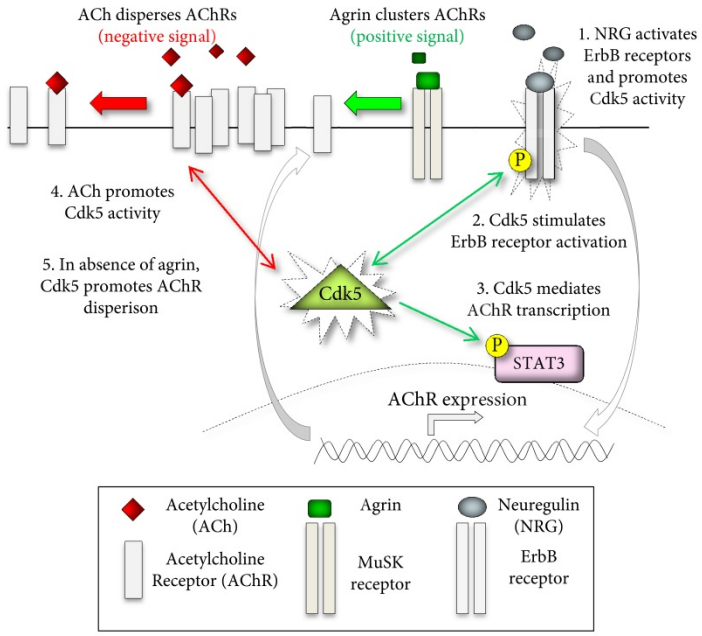

Figure 4 Cdk5 signaling at the postsynaptic neuromuscular junction. Cdk5 acts to both promote and suppress acetylcholine receptor (AChR) clustering during NMJ differentiation. Firstly, neuregulin (NRG) binds to its cognate receptor, which promotes Cdk5 activation at the synapse (1). Cdk5 activity potentiates activation of the pathway through phosphorylation of the ErbB-receptor (2) as well as the STAT3 transcription factor (3). Through these mechanisms, Cdk5 positively stimulates AChR transcription and NMJ development. Meanwhile, the main positive signal that stabilizes AChR clusters is agrin, and it is counteracted actively by acetylcholine (ACh). Interestingly, also ACh induces Cdk5 activity (4), which acts to disperse AChRs (5) that have not been stabilized by agrin. The signals that promote AChR cluster formation are depicted in green, while negative (dispersive) signals are highlighted in red.

1.3.4 Cdk5 actions in male and female reproductive organs

The testes contain a variety of cell types that are involved in sperm maturation. In seminiferous tubules, germ cells develop into motile haploid spermatozoa in a differentiation process termed spermatogenesis. Within the seminiferous tubules lie also the Sertoli cells that provide a structural support, secret sustaining hormones and provide nursery for developing spermatozoa. Sertoli cells are connected to each other through tight junctions, thereby separating the germ cells from the blood and creating the blood-testis barrier. Thus, similarly to the brain, the testis is a highly protected organ. In the interstitial space, located outside of the seminiferous tubules, lie the principal androgen-producing cells of the body, the Leydig cells. The androgen production in Leydig cells is mainly controlled by a complex feedback mechanism, which is ultimately orchestrated by the pituitary through secretion of the hormones Luteinizing hormone (LH) and Follicle-stimulating hormone (FSH).

Only a handful of studies have examined Cdk5 in the testicles. Cdk5 and p35 (but not p39) are expressed in the developing and adult testes in rodents and humans, mainly in Leydig and Sertoli cells (Musa et al., 2000; Rosales et al., 2004a; Session et al., 2001). Cdk5 protein is detected in the Sertoli (TM4) and Leydig (TM3) mouse cell lines, and shows histone-targeted, p35-dependent kinase activity in cells as well as in testes lysates (Musa et al., 1998; Musa et al., 2000; Rosales et al., 2004a). Interestingly, Cdk5 activity coincides with p35 expression and peaks at postnatal day 40 in rats, around the time when animals reach sexual maturity (Rosales et al., 2004a). Cdk5 activity was also found to correlate with serum, epidermal growth factor (EGF), LH, and human chorionic gonadotropin (hCG) stimulation of TM3 Leydig cells (but not TM4 Sertoli cells), possibly indicating that Cdk5 may have a role in Leydig cell proliferation, differentiation or testosterone production, which followed the treatments (Lin et al., 2009; Musa et al., 1998; Musa et al., 2000). The hCG-dependent testosterone production was counteracted by roscovitine treatment and Cdk5 small interfering RNA (siRNA), possibly through the protein Steroidogenic acute regulatory protein (StAR), which is involved in testosterone production (Lin et al., 2009). In addition to Sertoli and Leydig cell expression, Cdk5 is found in spermatids where it is thought to phosphorylate sperm tail structural proteins called outer dense fibers, at least in vitro (Rosales et al., 2004a; Session et al., 2001).

In the female reproductive organs, Cdk5/p35 is expressed and active in developing and adult ovary oocytes (Lee et al., 2004). It seems as Cdk5 does have some target proteins in the organ, as shown by phosphorylation of ovary lysates, but they remain yet unidentified.

1.3.5 Cdk5 in metabolic control

The pancreas is both an exocrine and endocrine organ that regulates the secretion of digestive enzymes and metabolic hormones in separate parts of the gland. The endocrine tissue is organized in specific structures called islets of Langerhans, which in turn are composed of a variety of cell types, including the insulin secreting β-cells and glucagon secreting α-cells. While insulin secretion lowers blood glucose, glucagon counteracts its effect in a delicately regulated signaling process that affects whole body metabolism.

Cdk5, p35 and p39 proteins are expressed in β-cells, where they partly co-localize with insulin (Lilja et al., 2001; Lilja et al., 2004; Ubeda et al., 2004). The proteins have not been described in other parts of the pancreas. The role of Cdk5 in insulin secretion has been a matter of debate, as it has shown both inhibitory and stimulatory effects on the glucose-stimulated insulin pathway; the discrepancies seem to derive from the use of different model systems, differences in experimental setup (glucose concentrations and treatment times), and possibly Cdk5 activator specificity. Early studies did suggest that Cdk5 activity is required for glucose-stimulated insulin secretion in β-cells isolated from both lean and obese mice (Lilja et al., 2001). Also, high glucose stimulation caused and increase in p35 levels and Cdk5 activity (Ubeda et al., 2004; Ubeda et al., 2006), which however, in this particular context may in fact link Cdk5 to high glucose induced β-cell apoptosis (glucotoxicity) rather than insulin secretion. Indeed, p25 generation, and the consequent hyperactivation of Cdk5, does induce caspase-3 cleavage, as has been independently demonstrated by Zheng and collaborators (2013). Exogenous p25 was, however, found to suppress insulin secretion (Zheng et al., 2013), highlighting that the experimental setup and the condition of the cells needs to be considered with care when interpreting data. Interestingly, p39 expression seems to induce general β-cells exocytosis more strongly than p35 expression (Lilja et al., 2004), suggesting that p35 is not the sole Cdk5 activator in β-cells.

On the other hand, many recent publications show that Cdk5 negatively regulates insulin secretion both in vitro and in vivo. Cdk5 phosphorylates the L-type voltage-dependent Ca^{2+} channel (L-VDCC) in β-cells, affecting its interactions with endogenous inhibitors (Wei et al.,

2005). If β-cell Cdk5 activity is inhibited with olomoucine, L-VDCC conductance capability is increased, with the consequence of boosted insulin secretion in glucose stimulated conditions (Wei et al., 2005). The cellular experiments were supported by experimental glucose-tolerance tests in the p35 knockout mouse, showing that KO mice have higher insulin levels after intraperitoneal glucose injection, and consequently lower blood glucose (Wei et al., 2005). Basal blood glucose levels were unchanged in the animals. Another study supporting the previous data, demonstrated that glucose-induced Sik2-kinase activation in β-cells caused phosphorylation-dependent degradation of p35, and an elevated insulin secretion (as did also Cdk5 inhibition) (Sakamaki et al., 2014). Moreover, p35 levels were reduced in islets isolated from high-fat diet fed mice (Sakamaki et al., 2014). Thus these in vivo-data convincingly support that Cdk5/p35 are important negative regulators of glucose-triggered insulin secretion, thereby affecting whole body metabolism. Meanwhile, Cdk5/p25 may preferably be involved in acute β-cell glucotoxicity.

In the 3T3-L1 adipocyte model, insulin treatment promotes Cdk5 activity (Lalioti et al., 2009; Okada et al., 2008). Both Cdk5 and p35 are expressed in white adipose tissue and differentiated 3T3-L1 adipocytes, where Cdk5 is involved in Glucose transporter type 4 (GLUT-4)-mediated and insulin-induced glucose uptake (Lalioti et al., 2009; Muruáis et al., 2009). Inhibition of Cdk5 activity with roscovitine or siRNA hampered glucose uptake (Lalioti et al., 2009; Muruáis et al., 2009), suggesting that Cdk5 is required for insulin-mediated glucose import. GLUT-4 is expressed only in insulin sensitive tissues, such as white fat and skeletal muscle. Insulin stimulates GLUT-4 expression at the cell membrane, allowing increased glucose uptake in the target cells. Some discrepancy exists in the literature describing the exact mechanism of adipocyte signaling, as Cdk5 siRNA is also reported to promote GLUT-4 membrane translocation induced by TNFα (Nohara et al., 2011; Okada et al., 2008). On the contrary, Muruáis and co-authors (2009) failed to detect a Cdk5-dependent difference in GLUT-4 intracellular localization in response to insulin.

1.4 Pathological deregulation of Cdk5

The correct regulation of Cdk5 activity at the right time and in the right place has proven to be crucial for normal cell function and prevention of diseases. Stress insults and other environmental- or intracellular stimuli affect the expression and turnover of Cdk5 and its activators, with the consequence of unfavorable Cdk5 hyperactivation (or sometimes hypoactivation), re-localization, and altered substrate specificity. p25-associated Cdk5 activity is indeed detrimental in many diseases of the brain, but the function of other activators in other tissues and disease contexts have remained more abstract, or have not always been questioned. Thus, Cdk5 is currently emerging as an attractive drug target in various diseases beyond the brain.

1.4.1 Neurodegenerative diseases and hyperactivation of Cdk5

Cdk5 has been strongly linked to the pathological neurodegeneration of a number of diseases, including Alzheimer´s disease (AD), Parkinson´s disease, Huntington´s disease and amyotrophic lateral sclerosis (reviewed in Patzke and Tsai, 2002b; Su and Tsai, 2011; Shukla et al., 2012). These diseases are characterized by elevated neuronal death, and despite the common disease prevalence, the mechanisms that contribute to the pathology are not completely understood due to their complex nature. A key factor that causes neuronal loss in these disorders is the accumulation of neurotoxic aggregates, such as amyloid-β plaques and neurofibrillary tangles in AD, generated by the aberrant activities of multiple kinases. In neurodegenerative pathogenesis, Cdk5 regulates both directly and indirectly the phosphorylation of such proteins that are involved in aggregate formation, including amyloid

precursor protein (Liu et al., 2003) and the microtubule stabilizing protein tau (Cruz et al., 2003; Noble et al., 2003; Piedrahita et al., 2010). In addition, Cdk5 is important for neurofilament (NF) transport in axons through a phosphorylation-dependent mechanism, which may in a disease context lead to NF aggregation (Ahlijanian et al., 2000; Rudrabhatla et al., 2010; Shea et al., 2004). Especially in AD, Cdk5 seems to act at many levels of disease progression through phosphorylation of several cytoskeletal targets, thereby contributing to the accumulation of both neurotoxic amyloid-β plaques and neurofibrillary tangles; the two hallmarks of AD-brain. Cdk5 does not act alone in neuronal pathogenesis, and it seems that a detrimental feed-forward loop of external cytotoxic insults promote Cdk5 neurotoxic activity even further (Lee et al., 2000).

It is well established that the calpain-mediated generation of p25, with the consequent deregulation of Cdk5, is in many ways neurotoxic (Ahlijanian et al., 2000; Lee et al., 2000; Patrick et al., 1999). While p25 generation is considered to be a direct cause of cell death in the abovementioned diseases, Cdk5/p35 does not seem to be capable of induction of apoptosis in neurons. Thus, calpain-mediated cleavage of p35 to p25, which can be induced by neurotoxic stimuli, ultimately determines whether Cdk5 acts as a neuron-sustaining kinase or a neuronal killer. Intriguing novel data indicates that p25 can, independently of Cdk5, bind to, activate and alter the substrate specificity of glycogen synthase kinase 3β (GSK3β) in neurons and thereby contribute to a neurodegenerative phenotype characterized by hyperphosphorylated tau and apoptosis (Chow et al., 2014). Recently, there has been significant interest towards the p10 fragment, which is generated from the p35 N-terminus during calpain cleavage. p10 has been shown to both promote (Zhang et al., 2012) and inhibit (Chew et al., 2010) Cdk5/p25-mediated apoptosis. The neuroprotective roles of various p35 peptide fragments have also been proposed to have an interesting clinical potential (Zheng et al., 2002; Zheng et al., 2010b).

1.4.2 Cdk5 in muscle pathology

Increased expression of Cdk5 or its activators have been associated with a few types of muscular diseases. Until now, there is no information whether the increased expression actually relates to an active role for Cdk5 in myopathic processes, or if the expression is simply reflecting a regenerating myogenic stage of the organ. Cdk5 has, for example, been detected in vacuolated myofibers in inclusion-body myositis (Nakano et al., 1999; Wilczynski et al., 2000). Interestingly, the disease resembles AD in that the degenerating myofibers contain AD-like hyperphosphorylated tau-inclusions that stain Cdk5 positive, possibly suggesting its involvement in the pathological progress of the disease (Wilczynski et al., 2000). In addition, increased p25 expression and Cdk5 activity have been reported in muscles from a mouse model for congenital endplate acetylcholinesterase deficiency (a type of myasthenic syndrome), which is characterized by loss of NMJ function (Zhu et al., 2013; Zhu et al., 2014). Denervation and necrotizing injury of skeletal muscle are also known to cause p35 expression and Cdk5 activity in the muscle, which most likely mimics a regenerative response that involves differentiation of muscle cells (Fu et al., 2002; Sahlgren et al., 2003). Thus, based on these few reports one cannot speculate further on an active role of Cdk5 in the generation of muscle pathology per se, but nonetheless, the kinase does seem to play some part in muscle healing and regeneration.

1.4.3 Type 2 diabetes and obesity: the Cdk5 connection

In type 2 diabetes (T2D) and T2D-associated obesity, Cdk5 has been found to promote the disease in at least two different cell types: in white adipose tissue and pancreatic β-cells. In a high fat diet-induced obesity mouse model, Cdk5 activity and p25 expression were shown to be induced in white adipose tissue after the mice were subjected to a 7-week long high fat diet (Choi et al., 2010). Interestingly, the Cdk5-mediated phosphorylation of peroxisome

proliferator-activated receptor γ (PPARγ) was required for the obesity-linked metabolic changes. Either Cdk5 inhibition with roscovitine or shRNA, or expression of the Cdk5 phosphosite-deficient S273A mutant PPARγ, increased expression of the anti-adipocytic adiponectin and adipsin that negatively regulate fat content (Choi et al., 2010). Hence, interrupting the Cdk5-mediated PPARγ phosphorylation has been considered as a novel anti-obesity strategy (Choi et al., 2011). PPARγ phosphorylation was additionally induced in adipocytes treated with cytokines TNFα and interleukin 6 (IL-6), suggesting that the obesity-provoked inflammatory response may drive the pathway described above (Choi et al., 2010). On the contrary, adipocyte-specific knockout of Cdk5 was found to cause a compensatory hyperactivation of ERK activator kinase 2 (MEK2)-ERK1/2-signaling, as well as PPARγ Ser273 phosphorylation by ERK1/2 (Banks et al., 2014). Intriguingly, due to the aforementioned mechanism, the high-fat diet fed adipocyte-specific Cdk5 knockout mice exhibit increased insulin levels and glucose intolerance (Banks et al., 2014), underscoring the two-faced actions of Cdk5 in the fat cell diabetic phenotype (Figure 5). Thus, the mechanisms that are controlled by Cdk5 in obesity are complex. Currently there is no further information on whether Cdk5 could contribute to maintenance of body homeostasis and glucose metabolism in other disease-related metabolic tissues, such as skeletal muscle.

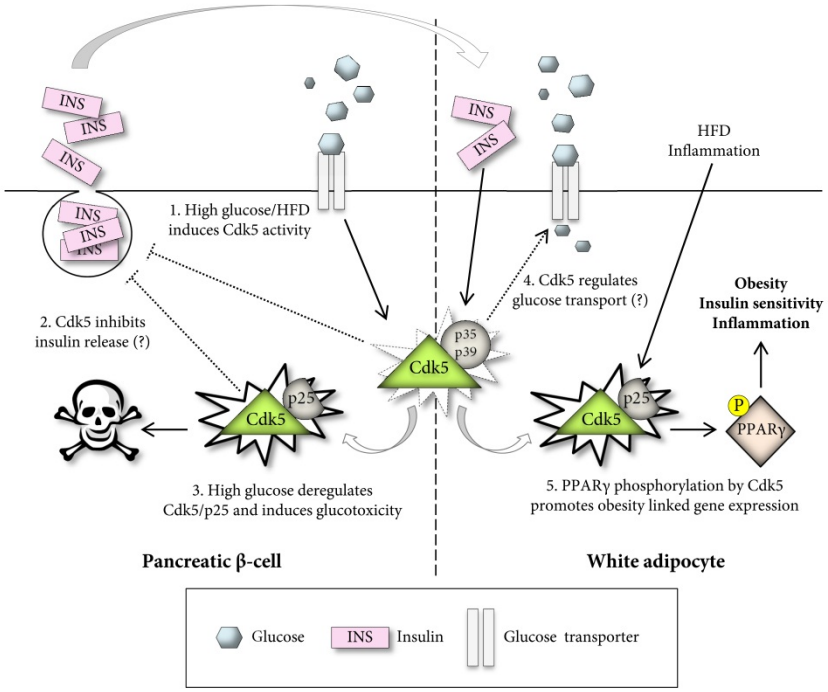

Figure 5 The diabetogenic actions of Cdk5 in T2D-associated pathology. The signaling functions of Cdk5 in diabetes are controversial. Accumulated data do point towards a pro-diabetogenic generation of Cdk5/p25. In pancreatic β-cells, high glucose induces Cdk5 activity (1), which in turn seems to inhibit insulin release, at least in vivo (2). While Cdk5/p35 seems to have a cytoprotective role under certain situations, high glucose causes generation of toxic Cdk5/p25, which is fatal for β-cells (3). Cdk5 activity is also induced by insulin in white adipocytes, where the kinase is involved in glucose transport (4). The generation of Cdk5/p25 can in turn be observed after high fat diet (HFD), or treatment of adipocytes with inflammatory cytokines. This excessive Cdk5 activity is involved in PPARγ phosphorylation and progression of diabetes through the increased expression of obesity-linked genes and promotion of insulin resistance (5). Thus, the data collectively suggests that while Cdk5/p35 has important physiological functions in both pancreas and fat tissue, the disease-induced Cdk5/p25 may have a critical role in generation of a diabetogenic phenotype in both β-cells and adipocytes. Therefore, an analogy in Cdk5 signaling can be drawn between neurodegeneration and diabetes.

Another aspect of T2D is the loss of function and eventual death of pancreatic β-cells. The hyperactivation of Cdk5 in β-cells with either exogenous p35 or p25 seems to cause high glucose-induced apoptosis in Min6 cells (Zheng et al., 2010c; Zheng et al., 2013). On the contrary, Cdk5 is also described to possess cytoprotective functions in rat insulinoma INS 832/13 cells (β-cell origin) and in isolated rat islets, where its downregulation causes increased apoptosis already in non-induced cells, but maximally upon islet treatment with amyloid polypeptide (Daval et al., 2011). In INS cells, Cdk5 or p35 downregulation caused a repression of the Focal adhesion kinase (FAK)-Akt survival pathway, which culminated in increased apoptosis. The contradicting results from Daval et al. (2011) and Zheng et al., (2010c, 2013) may be attributed to the use of different death-inducing stimuli and cell lines, or alternatively, by distinct functions of p35 and p25. Being cancer-derived, the cells may initially rely on different mechanisms to support proliferation and inhibit cell death. As in neuronal models, also the localization and proper amount of Cdk5 activity, which is regulated by the different activators, is critical for determination of Cdk5 kinase function; therefore both too little and too much activity may be unfavorable for β-cell survival signaling. Together these results illustrate the complexity of Cdk5 mechanisms in the pathogenesis of T2D, suggesting that it may regulate disease formation in several cell types simultaneously.

1.4.4 The multifaceted roles of Cdk5 in cancer

With the extending life-span of humans, we are confronted with an increasing amount of age-dependent cancer cases in the clinics. Different cancer types vary significantly in the molecular mechanisms that drive the uncontrollable proliferation of the cancer cells, the one property that initially caused malignancies to be classified into one disease category. Therefore, cancers actually seem to constitute a range of different diseases. Regarding their molecular characteristics and therapy options, we are in reality dealing with a broad spectrum of diseases that show variation not only at patient level, but also in terms of cellular heterogeneity within the tumor itself. According to Hanahan and Weinberg (2011), there are several key traits that portray cancer cell properties: the cells evade growth suppression, maintain cell proliferation, avoid immune destruction, deregulate cell energy metabolism, resist cell death, enable replicative immortality, activate invasion and metastasis, induce angiogenesis and accumulate mutations that stimulate genomic instability. The current consensus is that the tumor microenvironment and cancer-associated cells play a key role in tumorigenesis by allowing a cancer-permissive environment for the cancerous cells, including an inflammatory surrounding that drives oncogenesis (Hanahan and Weinberg, 2011). Thus, the cancer cells are not the sole targets for cancer therapy, as tumors are concurrently driven by cell-cell interrelationships and their dynamic environment over a long time that often spans an entire human lifetime.

Cdk5 has been connected with progression of at least melanoma (Abdullah et al., 2011), multiple (plasma cell) myeloma (Zhu et al., 2011), prostate cancer (Strock et al., 2006) and pancreatic cancer (Eggers et al., 2011), when evaluated by Cdk5 immunoreactivity in cancer patient samples. In addition, Cdk5 is significantly overexpressed at RNA level in breast, ovarian, lung, bladder and colorectal cancers, as well as in sarcomas, myelomas and lymphomas (Levacque et al., 2012). Cdk5 is also detected in Western blot samples from glioblastoma patients (Catania et al., 2001). The CDK5 promoter was found hypomethylated in mantle-cell lymphoma patients (Leshchenko et al., 2010), whereas CDK5 was found to be co-amplified with components of the epidermal growth factor receptor (EGFR)-Akt pathway in non-small cell lung cancer (Lockwood et al., 2008). Also polymorphisms in the CDK5 regulatory elements have been identified in lung cancer (Choi et al., 2009).

There are a few fundamental features in the molecular regulation of Cdk5 activity that challenge the analysis of its activation status in disease versus healthy tissue. Whereas several proteins are

over- or under-expressed in cancerous tissue, the expression levels of Cdk5 RNA or protein does not give an accurate picture of its functionality. As this is often the strategy of cancer tissue arrays, the involvement of proteins that are under complex regulation like Cdk5, may be underrepresented in such datasets. Therefore, the expression of its activators at protein level give a better understanding of the activity status of Cdk5, but even this approach seems to be obscured by the limited information on how the specific activators work in extra-neuronal tissue and how they are processed. In addition, there are no phospho-antibodies that could be used as reliable markers for Cdk5 activation, as is the case with many other well-studied kinases. As illustrated by prostate cancer, there is mixed information on the expression levels of Cdk5 in patient samples (Hsu et al., 2011; Levacque et al., 2012) that may have affected its appreciation as a feasible cancer drug target.

1.4.4.1 Regulation of cancer cell motility by Cdk5

As in neurons, where Cdk5 is an essential part of the signaling network that regulates migrating neurons´ cytoarchitecture, also cancer cells seem to utilize similar signaling pathways to drive migration, invasion and metastasis. In pancreatic cancer, K-Ras-mediated cell transformation promotes Cdk5 activity and migration through upregulation of p35, p39 and their cleavage products (Eggers et al., 2011; Feldmann et al., 2010). Most pancreatic tumors (67%) seem to have upregulated expression of Cdk5, p35 or p39, highlighting that the pathway is hyperactivated in patients (Eggers et al., 2011). Cdk5 is also important for invadopodia formation in head and neck squamous cell carcinomas (Quintavalle et al., 2011). In glioblastoma, Cdk5 is essential for cell migration and invasion through the stimulatory phosphorylation of Isoform A of phosphatidylinositol 3-kinase enhancer (PIKE-A), which acts upstream of the Akt-signaling pathway (Liu et al., 2008). Cdk5/p35 is also important for lung cancer cell migration (Demelash et al., 2012).

In breast cancer patients, Cdk5 and p35 levels are found to be upregulated and to correlate with higher tumor grade (Levacque et al., 2012; Liang et al., 2013). In fact, the proteins were also upregulated in transforming growth factor β-induced epithelial-mesenchymal transition of MCF10A cells, and Cdk5 was found to play an important role in the differentiation process through phosphorylation of FAK (Liang et al., 2013). Interestingly, Cdk5 has also been suggested to phosphorylate a microtubule-associated pool of FAK in melanoma cells that contributes to microtubule stability and mitosis (Rea et al., 2013), whereas phosphorylation of the same site in neurons affects microtubule-dependent migration (Xie et al., 2003). FAK seems to lie downstream of Cdk5 also in pancreatic cancer (Eggers et al., 2011).

1.4.4.2 Cdk5 in cancer cell proliferation and survival

Although Cdk5 activity is primarily linked to physiological differentiation processes, many cancer studies are supporting a novel role for Cdk5 in regulation of the cell cycle of malignant cells. For instance, Cdk5/p25 is crucial for medullary thyroid carcinoma formation in vivo, possibly through the phosphorylation of the tumor suppressor pRb, which promotes transcription of cell cycle regulators to promote proliferation (Pozo et al., 2013). Cdk5 has also been suggested to act through STAT3 in medullary thyroid carcinoma proliferation in cellular experiments (Lin et al., 2007). In SAOS-2 osteosarcoma cells, Cdk5 activity has on the contrary been implicated in an induced senescence cell model, where the phosphorylation of the actin-linked protein ezrin promotes the typical flattened senescent phenotype, and thus regulates actin polymerization through Rac-inhibition (Alexander et al., 2004; Yang and Hinds, 2006). A role for Cdk5 in senescence has not been established in other cell lines.

There is also accumulating evidence that Cdk5 functions as a pro-survival kinase in cancer cells. In leukemic Jurkat T-cells, Cdk5 was shown to phosphorylate Noxa on Ser13 upon glucose stimulation (Lowman et al., 2010). Noxa is a pro-apoptotic member of the Bcl-2 protein family, which is activated by p53 in response to DNA damage (Oda et al., 2000). Ser13 phosphorylation suppressed the apoptosis-inducing functions of Noxa and promoted cell survival (Lowman et al., 2010). The authors proposed an interesting hypothesis, where Cdk5 acts as a glucose-sensing master switch in lymphocyte cell survival. Cdk5 is also involved in T-cell activation and the following actin polarization in encephalomyelitis (Pareek et al., 2010).

Most studies support the fact that Cdk5 would sustain cancer progression by different mechanisms and there is less evidence on the roles of Cdk5 as a pro-apoptotic and anti-proliferative kinase in cancer. In HeLa cells, Cdk5 inhibition was suggested to promote cell survival, but direct evidence was lacking in both cases (Ajay et al., 2010; Kuo et al., 2009). In astrocytoma cells, Cdk5 overexpression promoted apoptosis of heat shocked cells, whereas expression of dominant negative Cdk5 did not (Gao et al., 2001). In the end, most studies support that Cdk5 promotes carcinogenesis through various pathways, many of them linked to cytoskeletal elements.

1.4.4.3 Prostate cancer: major signaling pathways and therapeutic challenges

Prostate cancer initially originates from the frequent age-dependent benign prostatic hyperplasia, which may eventually transform into prostatic intraepithelial neoplasia and further into full blown carcinoma (Figure 6). Thus, prostate cancer is most frequently diagnosed in men beyond 70 years. Main risk factors that contribute to disease development are age, race and familial susceptibility, but also dietary factors may facilitate disease progression. In Finland, prostate carcinoma is today the most common cancer type in the whole population with 4600 new cases registered in 2012 (Finnish cancer registry), which is nearly 30% of all male cancers. Of all cancer deaths in male patients, prostate cancer stands for almost 14% (or 855 in total). Prostate cancer has a high 5-year survival rate of 97%, but once it develops into castration-

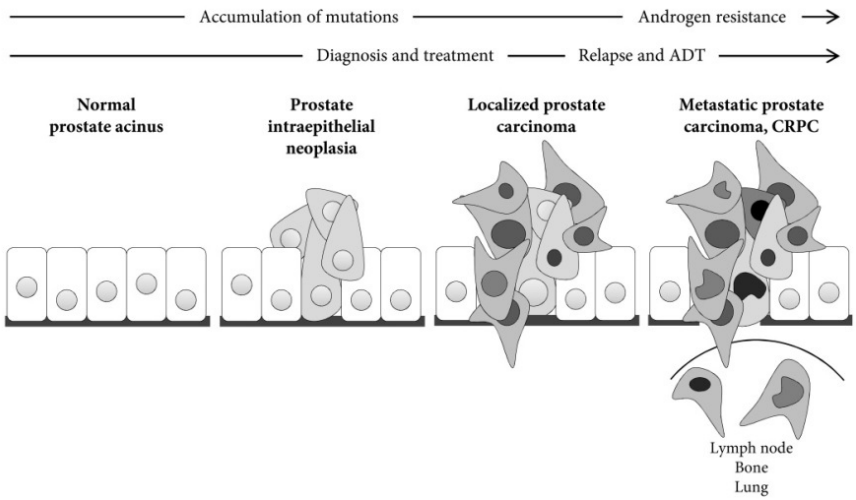

Figure 6 The development of prostate cancer. The pre-carcinogenic prostate hypertrophy and prostate intraepithelial neoplasia can, upon accumulation of mutations, develop into localized adenocarcinoma. Eventually the otherwise androgen dependent cells become androgen independent through mutations in the androgen pathway. At this stage the disease is termed castration resistant prostate carcinoma (CRPC), which cannot be treated. Prostate cancers are typically diagnosed at a stage when they are relatively harmless (localized prostate carcinoma), and can then be treated with prostatectomy. Eventually relapse may occur, to which the only treatment is androgen deprivation therapy (ADT). Unfortunately, ADT drives the development of CRPC, which is non-responsive to any treatment. Therefore, current therapy forms only stall disease progression and they eventually cease in effectiveness.

resistant prostate cancer, current therapies can only limitedly stall disease progression. Hence, there is a need to develop new therapies against the frequent and less aggressive androgen responsive carcinoma, to prevent its progression into castration resistant cancer. More importantly, there is an acute demand for novel drugs targeting the androgen independent state.

There are a few central signaling pathways that sustain prostate cancer proliferation (Figure 7). The androgen receptor (AR) is activated by cognate ligand binding (such as testosterone), and regulates prostate development, as well as secondary male gender characteristics. AR is amplified in 80% of prostate cancers and is thought to be the key regulator of prostate oncogenesis. As the AR is frequently amplified in prostate cancer, it is considered to be the main target for castration resistant tumor evolution, as it regulates a variety of pro-proliferative genes, anti-apoptotic players and promotes pathways required for invasion (reviewed in Green et al., 2012; Waltering et al., 2012). In addition, growth-promoting and apoptosis-inhibiting signaling cascades that are commonly found in a spectrum of cancers, are also deregulated in prostate cancer, including the PI3K-Akt and Ras-MAPK pathways, p53, c-Myc, pRb, etc. (Abate-Shen, 2000; Barbieri et al., 2013; Feldman and Feldman, 2001; Majumder and Sellers, 2005). For example, deregulation of the PI3K-Akt pathway occurs in 42% of primary prostate cancers, while mutations are found in 100% of metastatic cases, emphasizing the key role of this pathway in prostate cancer progression (Taylor et al., 2010). Further complexity of cancer signaling is achieved by cross-talk between pathways, as exemplified by partial functional compensation between AR and Akt (Carver et al., 2011; Thomas et al., 2013; Zhu and Kyprianou, 2008). In the case of inactivation of either pathway, feedback mechanisms can promote the activity of the other (Carver et al., 2011), while inhibition of both was shown to synergistically prevent tumor growth (Zhang et al., 2009). The Akt-AR interaction is, however, complex and not fully understood. The heterogeneity of prostate cancer mutations and the poor knowledge of patient stratification have hampered both the understanding of disease progression as well as prostate cancer drug development.

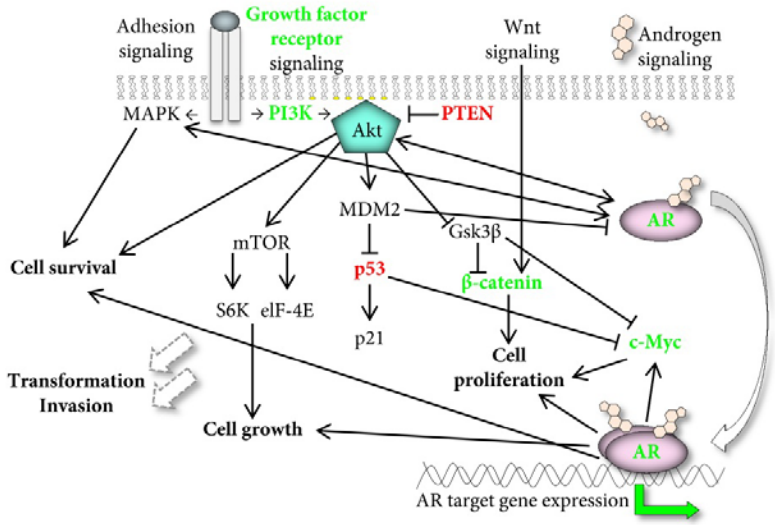

Figure 7 Central signaling pathways frequently deregulated in prostate cancer. One of the hallmarks of prostate cancer is the deregulated androgen signaling. AR is often amplified or mutated to allow ligand-independent activation or promiscuous activation by other hormones, or in other cases, AR co-factors are deregulated. Tumors can also sustain their own (intra-tumoral) hormone synthesis as an adaptation mechanism. Although AR drives many targets involved in cell proliferation, anti-apoptosis and migration, essential for prostate growth, also more general tumorigenic pathways (receptor tyrosine kinases, PI3K-Akt signaling, Wnt signaling) have been associated with prostate carcinogenesis. Commonly mutated tumor suppressors in prostate cancer are highlighted in red, whereas oncogenic proteins are in green.

1.4.4.4 Cdk5 signaling in prostate cancer

Implied by studies in other cancer models, Cdk5 has also been reported to regulate prostate cancer cell behavior. Inhibition of Cdk5 with either chemical inhibitors (roscovitine) or expression of dominant negative kinase strongly inhibits the migration and invasion of prostate cancer cells (Strock et al., 2006; Wissing et al., 2014). Decreased numbers of lung metastases in a xenograft mouse model agreed with cell-based results (Strock et al., 2006). Cdk5 also seemed to be important for tubulin polarization at wound edges in a cellular wound healing assay, suggesting that depletion of Cdk5 disturbs microtubule architecture (Strock et al., 2006). In a later study, Cdk5 targets were assessed in more detail, and Cdk5 was found to promote talin Ser425 phosphorylation in a metastatic subclone of PC-3 prostate cancer cells (Jin et al., 2014). Phosphorylated talin promoted β1-integrin activation and invasion both in vitro and in vivo, and reduced cancer cell susceptibility to anoikis (Jin et al., 2014). In SH-SY5Y neuroblastoma cells, the Cdk5-dependent talin Ser425 phosphorylation decreased proteasomal talin-degradation, increased optimal focal adhesion assembly and promoted cell migration (Huang et al., 2009a). Therefore, phosphorylation of talin by Cdk5 may be relevant in multiple diseases.

As previously discussed, one of the major pathways that contribute to prostate cancer proliferation is the AR-signaling axis. Experiments in LNCaP prostate cancer cells showed that Cdk5 promoted AR stabilization and Ser81 phosphorylation of the receptor (Hsu et al., 2011). Cdk5 also phosphorylates other members of the steroid hormone family, such as glucocorticoid receptor (Kino et al., 2007) and mineralocorticoid receptor (Kino et al., 2010), both of them resembling AR in terms of structure and activation mechanisms. The Cdk5-AR pathway is proposed to contribute to cell proliferation and prostate cancer xenograft growth (Hsu et al., 2011). Paradoxically, there is at least one publication reporting that Cdk5 does not phosphorylate AR Ser81 (Gordon et al., 2010). Both abovementioned reports are based on the use of phospho-specific antibodies. Instead, Cdk1 (Chen et al., 2006) and Cdk9 (Chen et al., 2012b; Gordon et al., 2010) are shown to phosphorylate AR Ser81, causing strong AR activation and chromatin binding. Given that Cdk5 can promote Cdk1 activity in neurons through phosphorylation of cell division cycle 25 phosphatases (Chang et al., 2012), the possibility for crosstalk between the Cdks should be considered when interpreting results, and special caution should be employed with use of pan-Cdk inhibitors. In addition, Cdk5 seems to promote STAT3 phospho-Ser727 immunoreactivity and target gene transcription in prostate cancer cells (e.g. junB, c-Myc), which could support a Cdk5-STAT3 signaling pathway in prostate cancer cell proliferation (Hsu et al., 2013). STAT3 Ser727 phosphorylation does additionally seem to correlate with prostate cancer grade (Qin et al., 2008).

Studies conducted in DU145 prostate cancer cells have on the other hand demonstrated that the cytotoxic activities of digoxin and retinoic acid induce calpain-mediated p25 cleavage, Cdk5 activation and apoptosis (Chen et al., 2012a; Lin et al., 2004). Downregulation or inhibition of Cdk5 decreases apoptotic marker expression, proposing that Cdk5 may relate to cell death signaling as well, but conceptually the results conflict with data described above, which portray Cdk5 as a tumor promoting kinase (Hsu et al., 2011; Hsu et al., 2013; Jin et al., 2014; Strock et al., 2006). The controversial results from prostate cancer model systems have left the Cdk5-regulated mechanisms debatable.

In many contexts, the complex regulation of signaling processes by Cdk5 has generated a large number of research papers, many of which are contradictory and require further investigation. The common use of high concentrations of roscovitine, especially in the cancer field, has hampered the credibility of many Cdk5-research papers, and they should be interpreted and re-cited with caution. Nevertheless, the view of Cdk5 functions is expanding. The intention of this review was to highlight its complex functions that have emerged in recent years, not only in

pathology, but also during tissue homeostasis. Exciting discoveries of novel Cdk5 activators have added to the knowledge of its complex regulatory mechanisms. Although Cdk5 and p35 were originally described to be found at highest levels in neurons, it has become apparent that even modest amounts of Cdk5 may be adequate for powerful and specific regulation of its target proteins. The work on Cdk5 activation mechanisms needs to be expanded in the future. Interestingly, in many cases Cdk5 and the mitotic Cdks appear to have similar substrates, although their functions rarely overlap temporally. Thus, Cdk5 could be considered to have evolved as a differentiation-linked Cdk also at a functional level. In many disease conditions, the Cdk5 hyperactivity gained by proliferating (cancer) cells or differentiated cell types drives inappropriate functions that were not intended for during tissue homeostasis.

2 The intermediate filament protein nestin in cell signaling

The cytoskeleton forms a core inside cells and is essential for cell motility, cell division and morphology. The mammalian cytoskeleton is divided into three structurally unrelated, and functionally different, classes; actin filaments (microfilaments), microtubules and intermediate filaments (IFs). In contrast to the early perception that the IF cytoskeleton only maintains cellular integrity and structure by acting as an immobile intracellular frame, it is today acknowledged to receive signals from outside cues, affect cellular signaling pathways and transmit or modify signals according to the cell´s needs. While protein kinases actively re-mold the cytoskeleton through the phosphorylation of cytoskeletal components and various regulatory proteins, the kinases are themselves subjected to regulation by their scaffolds in a continuous feedback loop.

2.1 Intermediate filament essentials: structure and dynamics

Compared to the generic actin and microtubules, which are found in all (eukaryotic) cell types, IFs show a cell type-specific expression pattern. The more than 70 members of IFs have evolved to perform highly specialized functions within their particular operative niche, and they are often conveniently used as markers for distinct cell types. Most metazoan cells have two separate IF networks; one in the cytoplasm, which is mainly accumulated around the nucleus, and the nuclear lamina, made up of the type V IF proteins lamins. Cytoplasmic IFs are important in organizing intracellular compartments and are vital components of desmosomes and other adhesive structures. The IF proteins (IFPs) are classified into subtypes (I-VI) according to sequence homology (Table 2). Although IFs vary in in terms of expression pattern, and share as little as 20% sequence homology among themselves, there is striking functional similarity between the different IF classes. Whereas actin and microtubule polymerization is a highly regulated energy-requiring process, IFs assemble spontaneously without the need of external energy. This feature stems from the secondary structure of the IF proteins (IFPs) and their apolar character. All IFs have a roughly similar central α-helical rod domain containing a repetitive pattern of hydrophobic amino acids that together with ionic interactions allow the formation of stable coiled-coil dimers of two IF monomers. The rod is subdivided into four domains that are separated by three non-α-helical linker regions, termed L1, L1-2 and L2. Many IFPs (such as nestin) cannot form homodimers in the initial head-to-head dimerization stage, but require the presence of another IFP type (such as vimentin and desmin in the case of nestin). Also keratins assemble through pair-formation between two different classes, while vimentin and desmin readily form homodimers. Two homo/heterodimers associate head-to-tail to form tetramers, which assemble into 16-dimer unit-length filaments and further into filaments, where the last step involves condensation of the structure to the final functional 10 nm thick filament. Although the rod domain contains the structural elements required for IF

polymerization, also the N-terminus is thought to play an important role in the actual filament formation by stabilizing the dimer structure. The most variable domains of IFs are in fact the N- and C-termini (heads and tails, respectively) that characterize the individual proteins. (Fuchs and Weber, 1994; Strelkov et al., 2003; Godsel et al., 2008; Herrmann et al., 2009.)

Table 2 Classification of Intermediate filament proteins and their associated diseases.

Class	IFPs	Expression	Associated diseases
Type I & II	Keratins	Epithelia, hair	EBS, hyperkeratosis
Type III	Desmin	Skeletal and cardiac muscle	Myopathy, cardiomyopathy
	GFAP	Astrocytes	Alexander disease
	Vimentin	Mesenchymal cells	Cataract
	Peripherin	PNS neurons	ALS
Type IV	Nestin	CNS progenitor cells, muscle	ALS, PD, CMT
	Neurofilament H, L and M	Neurons	
	Synemin α and β	Skeletal muscle	
	α-internexin	Neurons	
Type V	Lamin A/C	Most differentiated cells	Progeria, myopathy, cardiomyopathy
	Lamin B1 and B2	All eukaryotic cells	Lipodystrophy
Type VI	Phakinin	Lens	Cataract
	Filensin	Lens	

Abbreviations: EBS: Epidermolysis bullosa simplex, CMT: Charcot-Marie-Tooth disease, ALS: Amyotrophic lateral sclerosis, PD: Parkinson´s disease, GFAP: Glial fibrillary acidic protein. Source: Human Intermediate filament database, www.interfil.org (accessed 4.11.2014).

The importance of the IF cytoskeleton is underscored by the nearly 100 human diseases associated with IF mutations, some of which are summarized in Table 2. For example, several myopathic conditions of skeletal muscle and heart have been associated with desmin and lamin A/C mutations (Lu et al., 2011; Paulin and Li, 2004; Paulin et al., 2004). The lamin myopathy mutations can severely disrupt the stress-tolerance of myoblasts at the level of cellular signaling (Muchir et al., 2009; Muchir et al., 2013). Lamin A/C mutations are also implicated in the force endurance of cells, suggesting that the nuclear IFs may regulate the physical stress-tolerance of cells in myopathic conditions (Zwerger et al., 2013). Desmin on the other hand, is an important structural component of muscle found at Z-disks, NMJs, myotendinous junctions, as well as heart intercalated disks, and desmin null-mutation causes severe heart and skeletal muscle phenotypes, including cardiomyopathy and skeletal muscle weakness due to loss of muscle architecture and mitochondrial abnormalities (Li et al., 1997; Milner et al., 1996). Taking into consideration its role as a structural protein of mouse muscle, it is perhaps not surprising to find the many human myopathy mutations in the gene encoding desmin.

2.1.1 The IF protein nestin

Nestin is categorized as a class IV IFP due to similarities in intron positioning with the genes encoding neurofilaments (Dahlstrand et al., 1992a) and was first identified with the Rat-401 antibody in developing CNS (Hockfield and McKay, 1985). The rat nestin protein is 1805, mouse 1864 and human only 1621 amino acids long, and the C-terminus is the prime domain for inter-species variability (Yang et al., 2001). The human nestin protein is slightly shorter than in rodents, due to the loss of 11-amino acid repeats in the nestin C-terminus (41 vs. 18 copies in humans) (Dahlstrand et al., 1992a). Although the repeat copy number varies, the repeats themselves are highly conserved. The nestin N-terminus is curiously only 8 amino acids, while the tail is unusually long compared to other IFPs (1307 amino acids in humans). For comparison, the typical vimentin head/tail domains are 103/54 amino acids in length, respectively. Thus, the exceptional nestin tail, protruding from the filament structure, is accessible to PTMs and protein interactions. Nestin is conserved in mammals, but is found in both zebrafish (Chen et al., 2010) and birds (where it is termed transitin) (Jalouli et al., 2010; Wakamatsu et al., 2007), and both animals have been used as model systems to study nestin

functions in neuronal and muscle development. Opposed to transitin, zebrafish nestin is not expressed in developing muscles (Mahler and Driever, 2007). The closest human IF homologs to nestin is synemin, which has a similar short head/long tail protein structure (Guérette et al., 2007; Titeux et al., 2001). Synemin is enriched in skeletal muscle tissue where it polymerizes with desmin and vimentin (Titeux et al., 2001). Recently it was shown that synemin null mice show muscle hypertrophy and muscle membrane defects (Zhenlin et al., 2014). Also paranemin has been described as a high-molecular weight IF protein that resembles nestin by its rod-domain and long tail as well as its synemin-like expression pattern (Hemken et al., 1997), but it may actually be a splice variant of transitin.

Many IFPs, such as synemin (Xue et al., 2004) and GFAP (Middeldorp and Hol, 2011) have isoforms that are not only expressed in a tissue and developmental-stage specific manner, but also seem to have unique functions within the cells. Two nestin isoforms have been described so far: Nes-S and Nes-SΔ107-254, which are exclusively expressed in the otherwise nestin-negative dorsal ganglia (Su et al., 2013a; Wong et al., 2013). Nes-S incorporates into filaments in rat postnatal dorsal root ganglia sensory and motor neurons from postnatal day 5 (P5) onwards, and is not expressed elsewhere (including e.g. kidney and muscle tissue). Nes-S is alternatively spliced from the nestin gene so that exon 4, encoding part of the C-terminus, is lacking, giving rise to a protein of 49,5 kDa, with a 94 amino acid long tail containing a Nes-S-specific 32 amino acid tail domain (Su et al., 2013a). Nes-S-vimentin filament structures form in SW13 cells, while in dorsal root ganglia, Nes-S seems to form a filamentous network with the IFs peripherin and neurofilament-H (Su et al., 2013a). It is intriguing to speculate that Nes-S might have evolved in this particular cell type to perform nestin-like functions. Nes-SΔ107-254 on the other hand is missing most of its rod domain and cannot form filaments (Wong et al., 2013). Although present in dorsal root ganglia at low levels, it may lack a physiological function. An N-terminally GFP-tagged Nes-SΔ107-254 by Wong and colleagues (2013) did form cytotoxic aggregates when overexpressed, but this does not truly reflect a pathological function either.

Due to its short N-terminus, nestin needs another IFP partner to form filaments. Vimentin is thought to be the major polymerization partner of nestin in most cell types (Eliasson et al., 1999). In muscle cells, nestin co-polymerizes mainly with desmin (Sjöberg et al., 1994b), but interestingly, nestin distribution seems to be unaltered at NMJs and myotendinous junctions in desmin$^{-/-}$ muscle, suggesting that there are possibly other IFs compensating for desmin filament ablation in vivo (Carlsson et al., 1999). It should be noted that the desmin$^{-/-}$ NMJs are, however, more diffuse in their organization compared to wildtypes (Agbulut et al., 2001). Nestin can also form filaments in vitro with the neuronal IFP α-internexin (Steinert et al., 1999).

Although vimentin complements the structure that nestin is lacking in filament formation, also nestin seems to have a regulatory role in the heteropolymers. In vitro filament formation occurs optimally at a nestin:vimentin ratio of 1:4 (Steinert et al., 1999), and disruption of the ratio with high nestin concentrations has a negative effect on vimentin assembly. The same is true when nestin is overexpressed in mitotic cells: while overexpression of nestin shifts vimentin to a more soluble pool, nestin downregulation may promote vimentin filament formation around the mitotic spindle (Chou et al., 2003). However, nestin expression did not cause vimentin solubilization during cell division when the mitotic vimentin N-terminal phosphorylation site Ser56, required for vimentin filament reorganization, was mutated to alanine (Chou et al., 2003). Not only is vimentin required for nestin filament formation, but it seems like the nestin-vimentin interaction plays a role in regulation of vimentin organization through altered dimerization properties in vitro, as well as changed cellular signaling functions of vimentin. Thus, depending on the cellular context, nestin incorporation into major IF networks may drastically change the nature of the IF cytoskeleton in terms of functionality. Also nestin is

phosphorylated during mitosis on Thr316 by Cdk1, which alters its organization during cell division (Sahlgren et al., 2001).

2.1.1.1 Factors that control nestin expression

The nestin promoter is located in the 5´-untranslated region and its expression is mainly driven by the zinc-finger Sp1 and Sp3 transcription factors in most tissues (Cheng et al., 2004). Nestin expression is not regulated by upstream elements, but instead by enhancers in two of its three introns in a tissue-specific fashion. Nestin expression in the somites and myotome is regulated by the first intron, while the second intron regulates CNS-specific nestin expression (Kawaguchi et al., 2001; Zimmerman et al., 1994). The CNS-directed enhancer in the second intron of nestin is under the control of SOX- and Pit-1/Oct/Unc-86-family of transcription factors and contains hormone response elements that may add to the tissue specific fine-tuning of nestin expression (Josephson et al., 1998; Tanaka et al., 2004b).

Although the genetic regulation of nestin is crucial for its timely expression in specific tissues, nestin is, like most IFPs, strictly regulated at the post-translational level. The phosphorylation and ubiquitination of nestin (Sahlgren et al., 2001; Sahlgren et al., 2003; Sahlgren et al., 2006) allows its rapid reorganization in response to extracellular and intracellular stimuli. For example, nestin expression is under the control of stem cell-associated Notch during neuronal differentiation in glioma cells (Mellodew et al., 2004; Shih and Holland, 2006). In response to induction of differentiation, nestin is rapidly degraded by the ubiquitin-proteasome pathway (Mellodew et al., 2004). Notch signaling is by no means the only factor to regulate its expression, but also a variety of growth factors that inhibit or promote differentiation processes, regulate nestin. For example, nestin protein expression is under the control of the growth factors thrombin, EGF and platelet-derived growth factor and FGF-2 (Chang et al., 2013; Huang et al., 2008; Huang et al., 2009b). Also noteworthy is the strict regulation of nestin protein expression during muscle differentiation (Sahlgren et al., 2003; Vaittinen et al., 2001), which is immediately initiated upon growth factor withdrawal in cell culture systems. It is likely that both genetic and PTM-mediated stabilization occur simultaneously to regulate nestin protein levels in most systems. Depending on the context, the regulation of nestin may be direct, or alternatively, a response to initiation of a differentiation or de-differentiation program. It is not well understood what role nestin plays in the active maintenance of a progenitor-like state. Experiments in liver cancer tumorigenesis have revealed that nestin expression is regulated by p53-Sp1 signaling as an essential part of tumor initiation (Tschaharganeh et al., 2014), suggesting that nestin expression may in fact drive an oncogenic de-differentiation program in this particular setup, and proposing that nestin may be more than a mere marker of immature cells.

2.1.1.2 Dynamic expression of nestin during development and tissue pathology

Nestin is best known for its protein expression in neuroepithelial progenitor cells in the CNS and peripheral nervous system from early development (E7 neural ectoderm detected with GFP reporter; Kawaguchi et al., 2001), in a cell lineage that contributes to both neurons and glial cells (astrocytes, oligodendroglia). Nestin expression is not correlated with stem cell-like indefinite self-renewal and proliferation, but rather with a restricted proliferative capacity typical of progenitor cells (Wiese et al., 2004), which is why it is regularly used as a marker for those. In the embryonic muscle-forming somites, nestin LacZ-reporter activity (directed by intron 1) is apparent in E9 somite myotomes (Zimmerman et al., 1994), and nestin protein is detected from E9.5 (Kachinsky et al., 1994; Sejersen and Lendahl, 1993). Although nestin expression in the somites is directed by elements in the first intron, nestin intron 2-driven GFP reporter expression (Mignone et al., 2004) is detected in a few different progenitor cell types in

adult muscle (Birbrair et al., 2011; Birbrair et al., 2013b; Day et al., 2007). Using this (intron 2-directed) GFP mouse model, it was shown that the quiescence of muscle satellite cells goes strictly hand-in-hand with neuronal nestin-GFP reporter activity (Day et al., 2007). Nestin-GFP reporter expression in skeletal muscle also characterizes pericytes with myogenic precursor capacity, which contribute to muscle repair (Birbrair et al., 2013b), as well as neuronal lineage-committed precursors that resemble oligodendrocyte precursor cells (NG2-glia, which also express nestin protein) (Birbrair et al., 2011; Birbrair et al., 2013a). Nestin-protein is also expressed in the highly proliferative myoblasts (neuronal nestin-GFP negative; Day et al., 2007), which are expanding from satellite cells, as well as in differentiating myotubes (Kachinsky et al., 1994). In addition, nestin protein expression is induced when the adipogenic 3T3-L1 cell line is forced to differentiate into a myogenic lineage through exogenous expression of muscle transcription factors myogenin or MyoD (Kachinsky et al., 1994). Together these facts suggest that nestin expression is indeed highly linked to the muscle lineage. In the heart, nestin protein is transiently expressed in myocytes during E9-10.5 (Kachinsky et al., 1995).

Besides these major organ systems, nestin protein is expressed in some specific cell types during development and maturation, but has also been repeatedly reported in adult tissue resident progenitor cells (mostly mesenchymal stem cells; MSCs) that assist in tissue repair and renewal (Table 3, and references therein). It should be noted that the expression of nestin at protein level has not been carefully addressed in most research papers focusing on GFP reporter activity. The cases in Table 3 include examples where nestin protein has specifically been detected, unless indicated otherwise. Nestin protein is also expressed in certain myofibroblast populations, such as in breast myoepithelial cells, in the propria mucosa of colon and intestine, as well as in the endometrium (Kishaba et al., 2010). A more detailed description of nestin expression pattern is summarized in Table 3.

With the induction of tissue stress and injury, nestin protein has been found to be re-expressed at protein level at least in regenerating muscle after injury and in myodegeneration (Sjöberg et al., 1994a; Vaittinen et al., 1999), astrogliosis after CNS injury (Frisén et al., 1995), regenerating testes (during Leydig cell differentiation from vascular smooth muscle cells and pericytes) (Davidoff et al., 2004), healing kidney after nephritis (in mesangial cells and podocytes) (Daniel et al., 2008; Perry et al., 2007), as well as myocardium after infarction (Scobioala et al., 2008). Nestin is also upregulated in neoangiogenic blood vessels in re-molding tissue during development, injury and cancer (Mokrý et al., 2004). In addition to its expression in tumor vessels, nestin is found in many tumor cells, some of which are presented in Table 3. It is not clear whether nestin can be used as a cancer stem cell marker, although its expression in healthy tissue is associated with progenitor cell-like features. The expression of nestin in tumors has recently evoked plenty of interest due to its potential use as a clinical marker in certain cancers and as a possible therapeutic target. Nestin expression has been found to correlate with poor prognosis in at least melanoma and breast cancer (Ishiwata et al., 2011a), but the underlying function is incompletely understood.

2.2 Post-translational regulation of intermediate filament assembly

The prominent PTMs of IFs have in recent years been of utmost interest in the field, as virtually every member of the family is subjected to PTM. While PTM-forms such as ubiquitination is fundamental in regulation of IF protein levels, the PTMs additionally fine-tune the polymerization process of IF filaments, usually by increasing their solubility. The assembly of IFs is by no means a static process, but the turnover of filaments occurs constantly, although the proteins themselves might be stable proteins.

Table 3 Expression of nestin in various tissues.

Organ system	Specification	Physiological context	Pathological implications	References
Skeletal muscle	Satellite cells	Satellite cells (GFP reporter), myoblasts		(Day et al., 2007; Kachinsky et al., 1994)
	NMJ and MTJ	Constitutive expression	Denervation-regulated	(Carlsson et al., 1999; Vaittinen et al., 1999)
	Myotubes	Developing muscle	Regenerating myofibers	(Sejersen and Lendahl, 1993; Sjöberg et al., 1994a)
	Pericytes	Myogenic progenitors (GFP reporter)		(Birbrair et al., 2013b)
Cardiovascular	Cardiomyocyte	Developmental expression	Infarcted myocardium	(Kachinsky et al., 1995; Scobioala et al., 2008)
	Neovasculature	Smooth muscle and endothelial cells	Tumor angiogenesis	(Mokrý et al., 2004; Oikawa et al., 2010)
CNS	Neural stem cells	Common marker in early development	Correlation with various high-grade brain tumors	(Dahlstrand et al., 1992b; Dahlstrand et al., 1995; Lendahl et al., 1990)
	Astrocytes		Reactive astrocytes in CNS injury, astrocytoma	(Frisén et al., 1995)
Renal	Glomeruli	Podocytes (constitutive)	Kidney injury in podocytes, mesangial cells	(Daniel et al., 2008; Ishizaki et al., 2006; Perry et al., 2007)
Male reproductive organs	Testis	Developing (and less mature) Leydig and Sertoli cells	Testicular cancer	(Davidoff et al., 2004; Fröjdman et al., 1997; Lobo et al., 2004)
	Prostate		Prostate cancer (variably)	(Gu et al., 2007)
Female reproductive organs	Ovary		Ovarian cancer	(He et al., 2013)
	Mammary gland	Myoepithelial cells	Basal epithelial breast cancer	(Li et al., 2007; Liu et al., 2010)
Digestive system	Teeth	Tooth development and mature odontoblasts	Odontogenic tumors	(About et al., 2000; Fujita et al., 2006; Terling et al., 1995)
	Gastrointestinal tract		Expressed in various tumors	(Ishiwata et al., 2011a; Tsujimura et al., 2001)
Lung	(Myo)fibroblast		Injury-induced myofibroblast, lung cancer	(Chen et al., 2014; Kishaba et al., 2010; Ryuge et al., 2011)
Liver and pancreas	Liver	Mesenchymal progenitor cells	Stellate cells, hepatocellular- and cholangiocarcinoma	(Herrera et al., 2006; Niki et al., 1999; Tschaharganeh et al., 2014)
	Pancreas	Endocrine and exocrine precursor cells, stellate cells	Pancreatic cancer	(Bernardo et al., 2006; Lardon et al., 2002; Matsuda et al., 2011; Zulewski et al., 2001)
Endocrine organs	Adrenal cortex	Reticular layer	Cancer	(Lachenmayer et al., 2009)
Skeletal	MSCs	Osteoblast progenitors	Osteosarcoma	(Méndez-Ferrer et al., 2010; Ono et al., 2014; Veselska et al., 2008)
Hematopoietic	MSCs	Bone-marrow resident mesenchymal stem cells	Multiple myeloma	(Méndez-Ferrer et al., 2010; Svachova et al., 2011)
Adipose	MSCs	mRNA in isolated MSCs		(Timper et al., 2006)
Skin	Progenitor cells	Hair follicle and skin-derived progenitor cells	Skin cancers (e.g. melanoma)	(Akiyama et al., 2013; Flørenes et al., 1994; Li et al., 2003; Toma et al., 2005)
Sensory	Eye	Developing lens and retina, Müller cells		(Lee et al., 2012b; Yang et al., 2000)
	Taste	Fungiform papilla stem cells (GFP reporter)		(Mii et al., 2014)
	Ear	Progenitor cells in cochlea (GFP reporter)		(Kojima et al., 2004)
	Olfactory	Olfactory mucosa stem cells		(Tomé et al., 2009)

IFs are mainly phosphorylated on the head and tail domains, which protrude from the filament structure. The addition of phosphate groups dramatically affect the solubility of IFPs and mediates re-organization of the filament structure that is required e.g. during mitosis. For example, mitotic kinase activity is crucial for the solubilization of both cytoplasmic IFs, such as

vimentin (Yamaguchi et al., 2005), as well as the nuclear lamina (Peter et al., 1990). Also cell stress often causes prominent IF phosphorylation. As an example, keratin 8 can function in a cytoprotective manner in liver injury by attracting stress-kinase phosphorylation, a process which is thought to re-target harmful kinase activity away from apoptotic substrates (Ku and Omary, 2006). In addition, phosphorylation is important in the regulation of IF interactions. Also the significance of other PTMs (e.g. SUMOylation, glycosylation, acetylation, farnesylation), and their inter-dependence, is becoming more evident in the IF-field (Snider and Omary, 2014). Nestin has been found to be phosphorylated, ubiquitinated (Sahlgren et al., 2001; Sahlgren et al., 2003; Sahlgren et al., 2006) and glycosylated (Grigelionienė et al., 1996). To date, no other PTMs of nestin have been described in literature, but it would be surprising if nestin was not modified in a similar complex manner as other IFs. In addition, nestin antibodies often detect multiple differently migrating bands by Western blotting, suggesting the prominent existence of nestin splicing-, degradation- or PTM-isoforms.

2.2.1 Intermediate filaments as targets of Cdk5

Not different from other kinases, also Cdk5-mediated phosphorylation of IF networks affects the dynamics of IF assembly. Due to the close relation of Cdk phosphorylation motifs, many mitotic sites on IFs have been found to be regulated by Cdk5 during interphase. Thus, when a non-cycling cell meets a challenge that requires acute reorganization of the IF network, Cdk5 can be envisioned to substitute for the functions of classical Cdks in this context, allowing the cell to respond accordingly. Deregulation of Cdk5 activity can have serious consequences on IF assembly through the promotion of aggregate formation.

Neurofilaments

Cdk5, alike other members of the kinase family, phosphorylates multiple Lys-Ser-Pro (KSP) motifs on the C-terminus of neurofilament (NF) M and H isoforms (Lew et al., 1992b; Pant et al., 1997). The NF-H C- terminus contains over 50 KSP-repeats and is a heavily phosphorylated protein (Sun et al., 1996). The physiological importance of Cdk5 in regulation of NF localization was first demonstrated in the $Cdk5^{-/-}$ mouse model (Ohshima et al., 1996), and later, the phosphorylation has indeed been shown to regulate axonal transport of the IFs (Ackerley et al., 2003; Shea et al., 2004). Deregulated Cdk5 is believed to cause NF hyperphosphorylation in many neurodegenerative diseases, such as Parkinson's disease (Nakamura et al., 1997), amyotrophic lateral sclerosis (Nguyen et al., 2001), and Niemann-Pick type C disease (Bu et al., 2002), which has a neuronal pathology that resembles AD. Most Cdk5-related neurodegenerative phenotypes are, however, not single-handedly caused by NF hyperphosphorylation.

Nestin

A series of reports have described the interplay between nestin and Cdk5. Identified by in vitro phosphorylation and mass spectrometry, Cdk5 was shown to phosphorylate nestin on Thr316 and Thr1495, which is involved in remodeling of the nestin cytoskeleton (Sahlgren et al., 2003). Additionally, Thr316 is phosphorylated by Cdk1 during mitosis, causing reorganization of nestin filaments (Sahlgren et al., 2001). Also nestin Ser1837 phosphorylation (a Cdk5 consensus site) was reported to be downregulated in E18.5 $Cdk5^{-/-}$ brain in a phosphoproteomic-study, possibly implying it may be a Cdk5 site in vivo (Contreras-Vallejos et al., 2014). The phosphoregulation of nestin has vast consequences on Cdk5 itself, and the detailed signaling mechanism is discussed further in forthcoming chapters.

Lamins

Lamin phosphorylation during cell division is essential for disruption of the physical nuclear lamina, allowing proper chromosome segregation to occur (Heald and McKeon, 1990; Peter et al., 1990). Some major mitotic sites (e.g. Ser22, Ser392) have also been found to exist in a phosphorylated status during interphase (Kochin et al., 2014), and thus, they are believed to be crucial in determination of lamin solubility. In a neuronal model of glutamate- or amyloid-β-induced apoptosis, Cdk5 was suggested to promote phosphorylation of lamin A on Ser392 and Ser22 (as well as of the equivalent sites on lamin B1) (Chang et al., 2011). Chang and colleagues (2011) postulated that upon neurotoxic insults, the hyperactivation of Cdk5 kinase activity drives the solubilization of the nuclear lamina as an early step of neuronal apoptosis. In fact, mutation of Ser392 to alanine did reduce neurotoxicity, suggesting that lamins are important targets of hyperactivated Cdk5, and that the nuclear dispersion is not solely a consequence of cell death (Chang et al., 2011).

Vimentin

Vimentin Ser56 was originally found to be phosphorylated by Cdk1 during mitosis, allowing rearrangement of vimentin filaments during cell division (Chou et al., 1991). Upon artificial neutrophil activation, Cdk5 was shown to phosphorylate vimentin on Ser56, causing a polarization of the vimentin cytoskeleton (Lee et al., 2012a). The phosphorylation-mediated remodeling of vimentin was proposed to be the key factor for Cdk5-dependent neutrophil secretion (Lee et al., 2012a; Rosales et al., 2004b).

2.3 Intermediate filaments as signaling scaffolds

Not only is the IF cytoskeleton constantly being remodeled as a response to the cell differentiation stage, but the solubility, altered intracellular localization and organization have dramatic effects on IF interaction partners. In recent years, it has come to the attention of the field that the IFs actively regulate functions and positioning of organelles, and are involved cell migration, adhesion, differentiation and mitosis; processes which are highly dependent on the flexibility of the cells´ cytoskeleton. Many IFs are crucial for the stress tolerance of cells when they are challenged to a particular strain.

Because of their strict differentiation-regulated expression pattern, it is perhaps not completely unexpected that IFPs act at the gates of differentiation processes. The differentiation of muscle is an interesting example, as the different stages are characterized by the dynamic expression of many IFPs. Myoblasts lacking lamin A/C differentiate at a slower rate due to the decreased expression of the myogenic determinant MyoD (Frock et al., 2006). Nucleoplasmic lamin A/C-lamina-associated polypeptide 2α (LAP2α)-pRb complexes suppress proliferation and enhance differentiation of at least adipocytes and muscle stem cells at initial stages of commitment (Dorner et al., 2006; Gotic et al., 2010). Thus, the lamin A/C-regulated differentiation of muscle cells is likely to be under strict control of not only lamin protein levels, but by the solubility state of lamins. As the PTMs are essential for regulation of lamin solubility, it would be highly interesting to assess the function of lamin phosphorylation during muscle differentiation. As another example, the genetic depletion of synemin increases the myogenic commitment of muscle stem cells (Zhenlin et al., 2014). Desmin, in turn, does not seem to play an essential role in muscle differentiation or fusion in a knockout-approach (it is likely that some its functions can be substituted by other similar proteins), but the organization of the sarcomeric structure of muscle (Li et al., 1997; Milner et al., 1996), as well as organelle distribution, are severely devastated. Conversely, the transient downregulation of desmin inhibits myoblast differentiation very potently, suggesting that desmin is nevertheless required for earlier processes of muscle differentiation (Li et al., 1994). Together these results illustrate the

importance of IFPs and their solubility state in regulation of myogenic differentiation. Naturally, this is not the only differentiation model where IFPs are essential for cell fate determination. The roles of IFPs in regulation of the proliferation/differentiation switch are of special interest in the context of cancer.

2.3.1 The physiological functions of nestin

The function of nestin has remained somewhat unclear, with the only very recent generation of three distinct mouse models lacking nestin. The first one to be published targeted the coding region of nestin exon 1 by homologous recombination and the mice showed perinatal lethality (Park et al., 2010); a relatively uncommon, but not unheard of, phenotype in the field of IF knockout models. These nestin knockout mice died due to high apoptosis-rate of neuronal precursors during development (Park et al., 2010). Later, Mohseni and colleagues (2011) published a viable and grossly normal nestin knockout mouse, where most of exon 1 and part of the 5´-untranslated region were deleted by gene targeting. The knockout mouse by Mohseni et al. (2011) did, however, show aberrant NMJ structure and motor coordination problems. Both knockouts were in C57BL/6 background (Mohseni et al., 2011; Park et al., 2010). In line with Mohseni et al. (2011), a third nestin deficient mouse was generated by ubiquitous lentiviral expression of nestin shRNA plasmid, causing a disruption of NMJ organization in nestin downregulated mice (Yang et al., 2011). Also the shRNA mouse was viable. As two independent mouse strains lacking nestin were viable, and because the deletions of minor IFPs are rarely deadly, it seems probable that the embryolethal knockout model generated by Park and coauthors (2010) may have suffered from some unidentified defect, which eventually may have caused the observed lethality.

As nestin expression is generally linked to highly proliferative tissues, such as developing neuroepithelium and several types of cancer, there has been an interest to understand the role of nestin as a regulator of progenitor cell proliferation. One interesting aspect was provided by Reimer and colleagues (2009), when they reported an interplay with the steroid hormone receptor family member glucocorticoid receptor (GR) and the nestin-vimentin IF network. They proposed that this IF cytoskeleton is important for sequestration of the glucocorticoid receptor to the cytoplasm: depletion of nestin promoted nuclear accumulation of the GR, while its overexpression resulted in GR cytoplasmic sequestration (Reimer et al., 2009). As the shRNA-mediated downregulation of nestin correlated with cell cycle arrest, the authors suggested that in the absence of nestin, the growth inhibitory effects of nuclear GR were amplified. Nestin and GR co-expression has also been reported in melanoma patient samples (Lai et al., 2013).

The nestin/vimentin-scaffold also regulates the activity of the ubiquitous insulin degrading enzyme (IDE). Besides insulin and amyloid-β, also ubiquitin belongs to the substrates of IDE (Ralat et al., 2011). IDE bound specifically to the soluble pool of the nestin-vimentin cytoskeleton in a phosphorylation-dependent fashion, but also separately to the nestin tail fragment 641–1177 in vitro (Chou et al., 2009). Soluble nestin-vimentin reduced IDE-activity towards insulin and ubiquitin, while stimulating degradation of the small substrate peptide V (Chou et al., 2009; Ralat et al., 2011). Together these results indicate that the solubility of the nestin-vimentin filaments control the activity and substrate specificity of this enzyme. Taking into consideration the numerous and highly potent substrates of IDE, the IF phosphorylation status can have extensive effects on cell physiology.

Studies on transitin, the bird ortholog of nestin, have revealed roles in both proliferating bird myoblasts and neuroepithelial cells. While knockdown of transitin in chick myoblast prevents differentiation and promotes proliferation (Jalouli et al., 2010), transitin knockdown in

neuronal progenitors promotes neuronal differentiation at the expense of proliferation (Wakamatsu et al., 2007). In fact, transitin binds to the Notch inhibitor Numb and directs its asymmetrical division during mitosis of neuronal progenitors, which may underlie the observed effect of transitin knockdown on neuronal progenitor fate (Wakamatsu et al., 2007).

2.3.2 Interplay between Cdk5 and nestin in muscle models

Nestin and Cdk5 were first shown to interact in the rat-derived neuronal progenitor cell line ST15A (Sahlgren et al., 2003). Expression of Cdk5/p35 strongly promoted nestin phosphorylation on Thr316, but several phosphosites that remained unidentified, seemed to be induced (Sahlgren et al., 2003), suggesting that nestin is heavily phosphorylated by the kinase. The nestin-Cdk5 interaction was confirmed to occur also in differentiating myoblasts, where both nestin protein expression and Cdk5 activity are induced strongly at later stages of differentiation (Sahlgren et al., 2003). The organization of nestin during muscle differentiation is highly dynamic, when the typical perinuclear nestin found in proliferating cells reorganizes into long filaments that follow the shape of the elongated and fused myotubes (Sahlgren et al., 2003). The transport of nestin (and other muscle IFs) to the tips of myotubes is regulated by the kinesin-motor protein family member Kif5b during myoblast differentiation (Wang et al., 2013), but the reorganization is otherwise phosphorylation-dependent (Sahlgren et al., 2003). Phospho-Thr316 on nestin was found to exist exclusively in soluble fractions in differentiating myoblasts, suggesting that Cdk5-mediated nestin phosphorylation releases a pool of soluble nestin. The strong induction of nestin protein levels and filament re-organization during differentiation are also dependent on Cdk5 activity (Sahlgren et al., 2003). Therefore, there were clear indications of Cdk5-mediated regulation of nestin organization in myocytes. Interestingly, the inhibition of Cdk5 with roscovitine increased the association between nestin and p35, suggesting that Cdk5 activity regulates the scaffolding properties of nestin towards its own activator. Later, PKCζ, which is an essential upstream regulator of Cdk5 activity, was also demonstrated to be important for nestin filament organization and induction of nestin protein levels during the process, as Cdk5 remains essentially inactive in the absence of functional PKCζ (de Thonel et al., 2010).

In mature muscle, nestin is localized directly underneath the postsynaptic plasma membrane of the NMJ (Vaittinen et al., 1999; Vaittinen et al., 2001), where it extends into sarcomeres in the vicinity of the NMJs and surrounds the junctional myonuclei (Kang et al., 2007). Nestin expression at the postsynaptic NMJ is regulated by innervation, causing nestin mRNA to be transcribed in the synaptic myonuclei (Kang et al., 2007). Although the regulation of nestin in muscle is not completely understood, it seems as nestin expression is regulated by several factors.

As nestin is present at NMJs, and co-localizes with the postsynaptic AChRs together with Cdk5 (Sahlgren et al., 2003), it is perhaps not surprising that nestin-mediated scaffolding of Cdk5 is essential in determination of its kinase activity at the NMJs. As discussed previously, Cdk5 acts at several levels of NMJ development to regulate both positive (NRG-induced AChR transcription) and negative signals (ACh-mediated Cdk5 activity disperses AChR clusters, which are not stabilized by agrin) that act on AChR clustering (Fu et al., 2001; Fu et al., 2005; Lin et al., 2005). As a response to ACh agonists, C2C12 myotubes upregulate Cdk5 activity, induce p35 membrane recruitment and consequently trigger nestin Thr316 phosphorylation (Yang et al., 2011). Downregulation of nestin, or expression of phospho-deficient T316A mutant nestin, causes suppression of Cdk5 activation and a reduction in ACh agonist-dependent NMJ dispersion, showing that (phosphorylated) nestin is required for Cdk5-mediated AChR dispersion (Yang et al., 2011) (summarized in Figure 8). Nestin shRNA mice that were generated by Yang et al., (2011) did show an increase in AChR cluster number, indicating

that nestin is required for optimal NMJ dispersion. The results were supported by a genetic nestin deletion mouse model, which similarly to the shRNA mice, showed an increase in AChR cluster number and size, causing motor coordination problems in the nestin deficient mice when measured by the Roto-Rod test (Mohseni et al., 2011).

2.3.3 Cdk5 and nestin in neuronal cell death and survival

A cytoprotective role for nestin was first demonstrated in the neuronal ST15A cells (Sahlgren et al., 2006). When the cells were treated with hydrogen peroxide to induce apoptosis, nestin was found to undergo proteasomal degradation at the time of cell death, and intriguingly, downregulation of nestin increased apoptosis further, while expression of exogenous nestin had a strong cytoprotective effect (Sahlgren et al., 2006). Cdk5 turned out to be the executor of apoptosis in this particular model, and expression of DN-Cdk5 rescued the increased apoptosis of nestin-downregulated cells (Sahlgren et al., 2006). In fact, exogenous expression of nestin protected p35 from ubiquitination and proteasomal degradation, while reducing its calpain cleavage to p25 during apoptosis. Studies that were based on expression of N-terminally green fluorescent protein (GFP)-tagged nestin truncations showed that the nestin tail contributes strongly to its own degradation; the nestin truncation containing amino acids 1-640 of rat nestin (Nest-640) is considerably more stable compared to full length forms (Sahlgren et al., 2006). Immunoprecipitation studies also revealed that Cdk5/p35 binds to the nestin C-terminus (between amino acids 314-640) next to the rod domain (Sahlgren et al., 2006). Finally, Sahlgren and colleagues (2006) showed that while expression of Nest-640 did not increase steady-state p35 protein levels as such, its intracellular localization (and thus Cdk5 activity) shifted towards a soluble pool as determined by cellular fractionations. Thus it is believed that a soluble pool of Nest-640, which exists in significant amounts in the transfected cells, sequesters Cdk5 activity to the cytoplasm and protects p35 from ubiquitination, thereby preventing cytotoxic Cdk5/p25 activity in the nucleus. Importantly, the nestin-Cdk5 interplay occurred in both directions: while nestin was shown to regulate p35 turnover, disruption of Cdk5 activity with roscovitine or DN-Cdk5 significantly retarded the H_2O_2-induced nestin degradation (Sahlgren et al., 2006). As Cdk5-mediated nestin phosphorylation promotes filament solubilization (Sahlgren et al., 2003), the evidence from both neuronal and muscle systems imply that Cdk5 itself acts to regulate the turnover of p35 through nestin, to prevent unwanted kinase activity. The signaling events at the NMJ and during apoptosis are recapitulated in Figure 8.

The novel Nes-S isoform was also seemingly interacting with Cdk5 when overexpressed in N2a neuroblastoma cells that are intrinsically Nes-S deficient (Su et al., 2013a). In support of nestin-Cdk5 interplay in neurons, expression of Nes-S promoted survival after H_2O_2 treatment (Su et al., 2013a). In addition, Nes-S downregulation seemed to impair primary dorsal root ganglia viability. Although further evidence is required to understand the interplay between Nes-S and Cdk5, these results nicely support the nestin-Cdk5 interaction, which has been under intense investigation in muscle and neurons previously. Also in zebrafish, the downregulation of nestin causes an increase in apoptosis of neuronal precursors during development of brain and eye, leading to severely malformed and small-sized brains and hydrocephalus (Chen et al., 2010). In rat vascular smooth muscle cells nestin downregulation enhances H_2O_2-induced apoptosis similarly to the ST15A model system, but the link to Cdk5 remained unclear from this particular study (Huang et al., 2009c). Results suggested that Cdk5 may have acted as an anti-apoptotic kinase instead in this particular model system, leaving the details unclear.

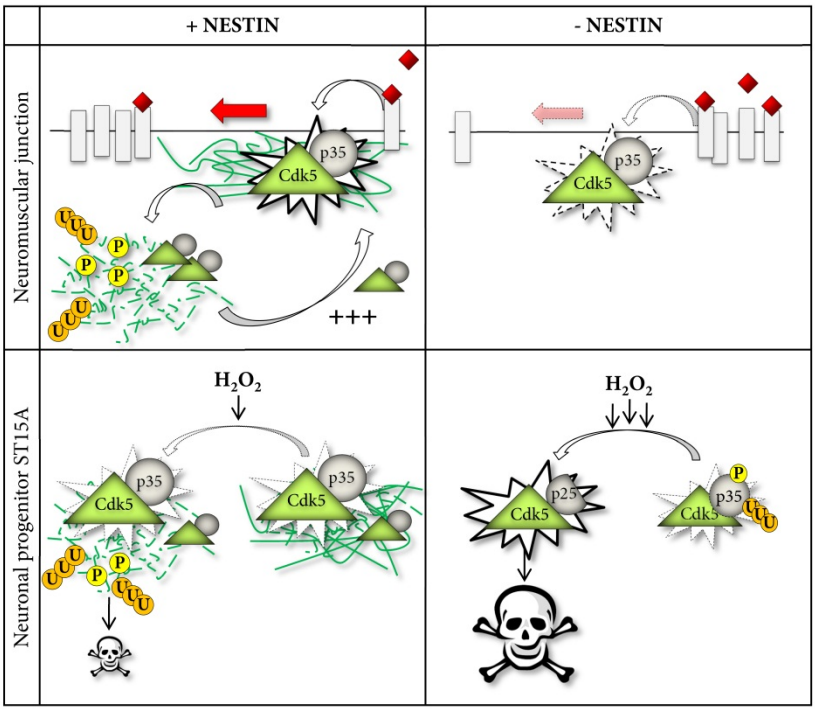

Figure 8 The role of the nestin scaffold in the regulation of Cdk5 signaling. In a model by Yang et al. (2011) and Mohseni et al. (2011) (upper panel), nestin is required for Cdk5 activation at the NMJ membrane, where Cdk5 kinase activity acts to disperse non-stabilized AChRs. ACh itself drives Cdk5 activation. Activated Cdk5 phosphorylates nestin, which stabilizes Cdk5/p35 transiently at the membrane. Eventually, the phosphorylated nestin is degraded. If nestin is completely removed from the system, Cdk5 is not activated as efficiently, resulting in increased AChR clustering, as observed in the nestin deficient mice. A similar regulatory scaffold determines Cdk5-mediated neuronal apoptosis (Sahlgren et al., 2006) (lower panel). In the presence of nestin, oxidative stress induces Cdk5 activity, which is targeted towards the nestin filaments. The consequential phosphorylation-mediated nestin solubilization protects Cdk5/p35 at the expense of Cdk5/p25. Eventually, nestin is degraded. In the case nestin is removed by shRNA, p35 is uncontrollably processed and Cdk5/p25-generation is favored, resulting in increased apoptosis when the death-inducing Cdk5 activity is unleashed.

2.3.4 The role of nestin in podocyte signaling

As highlighted before, Cdk5 activation by p35 and/or cyclin I is important for renal podocyte survival through the maintenance of adequate levels of anti-apoptotic proteins (Brinkkoetter et al., 2009; Griffin et al., 2006; Taniguchi et al., 2012). In transgenic mice lacking either p35 or cyclin I, podocyte apoptosis was increased compared to WT mice during podocyte stress, and kidney function was further impaired in the double knockout mice (Brinkkoetter et al., 2009; Brinkkoetter et al., 2010; Taniguchi et al., 2012), proposing that the activators could partly compensate for each other´s functions, although Cdk5 may have altered substrate specificity depending on which activator it is bound to. Baseline kidney function and podocyte apoptosis was normal in all mice. Also a pro-apoptotic role for Cdk5 in high glucose-treated cultured podocytes has been suggested in opposition to the abovementioned observations (Zhang et al., 2014). In a streptozotocin-induced model of rat diabetic nephropathy, Cdk5 expression was found to be induced in injured glomeruli (Liu et al., 2013). Furthermore, Cdk5/p35 expression and activity was promoted by high glucose treatment, but apoptosis was found to be only modestly inhibited in the presence of relatively high concentrations of roscovitine (50 μM) or specific Cdk5 targeting (Liu et al., 2012; Zhang et al., 2014).

Intriguingly, nestin is also expressed in fetal and adult podocytes, where it co-localizes with Cdk5 in immunostainings (Bertelli et al., 2002). In the adult organism, podocytes are one of the

few places where nestin and Cdk5 are strongly expressed under homeostasis and can be studied jointly, implying that together they may have highly specialized functions in that particular cell type. In fact, downregulation of nestin in cultured podocytes caused a modest increase in the apoptotic population (Liu et al., 2012), suggesting that it may have neuron-like cytoprotective scaffolding functions for Cdk5 during podocyte apoptosis (Sahlgren et al., 2006). The nestin-Cdk5 signaling axis has not been studied in further detail in renal systems. Nestin expression levels are known to respond to podocyte injury in different injury models and kidney disease patients, but both up- and downregulation has been observed, probably due to different models used and differences in the degree of injury (Su et al., 2007; Wagner et al., 2006; Zou et al., 2006). Nestin is also expressed in intraglomerular mesangial cells (specialized pericytes) upon specific injury towards these cells (Daniel et al., 2008). When depleted, the mesangial cells proliferate and repopulate their niche and depletion of nestin with siRNA suppresses their proliferation to some extent (Daniel et al., 2008).

2.3.5 Nestin - more than a marker in cancer

Nestin is expressed in a variety of cancers. The downregulation of nestin seems to negatively affect the cell migration/invasion of at least pancreatic cancer cells (Matsuda et al., 2011), glioma cells (Ishiwata et al., 2011b), lung cancer cells (Takakuwa et al., 2013) and melanoma cells (Akiyama et al., 2013). In prostate cancer cell models, two separate studies have questioned the role of nestin in migration and invasion. While Kleeberger and colleagues (2007) found that nestin downregulation inhibited migration and invasion, a later study argued that nestin downregulation promotes invasion through regulation of focal adhesion component turnover and integrin membrane expression (Hyder et al., 2014). The downregulation of nestin also promotes melanoma cell invasion and matrix-metalloprotease upregulation (Lee et al., 2014). Some reports suggest that nestin expression might be linked to epithelial-mesenchymal transition, at least in pancreatic (Su et al., 2013b) and breast (Zhao et al., 2014) cancer cell models. In many types of cancer cells the knockdown of nestin has been found to interfere with cell growth and viability, including lung cancer cells (Takakuwa et al., 2013), liver and breast cancer cells (Tschaharganeh et al., 2014; Zhao et al., 2014) and in melanoma (Akiyama et al., 2013). These, and many other publications, do support that nestin plays a role in tumorigenesis and cancer cell motility, but the effects are cancer cell line-specific and the mechanisms are in many cases not understood well, if at all. Also the aspects of nestin as a potential regulator and target for tumor angiogenesis (Ishiwata et al., 2011a; Matsuda et al., 2013) have showed growing interest in the past years, as nestin is highly expressed in pericytes and endothelial cells in neovasculature.

The specificity of nestin expression in mesenchymal stem cells, neuronal progenitors and cancer have created an interest to understand its expression pattern in more detail, possibly even including its use as a clinical marker. There are, however, only a limited number of research articles that address in detail the functionality of nestin and the significance of its presence in tumors. Given its surprisingly abundant, but highly specific expression, which has expanded in recent years, there is an increasing need to understand better the molecular functions of nestin.

3 Myogenesis

Skeletal muscle contributes to approximately 40% of the total body weight in mammals. Limb skeletal muscles originate from the embryonic somites of mesodermal origin, which reside in pairs on both sides of the developing vertebrae. The somites are sites of myoblasts expansion, from where the myogenic cells migrate to the myotome, where primitive skeletal muscle tissue

is formed. First somites appear around E8 in mouse development, after which they multiply to a final number of more than 60 somite pairs. During the three first postnatal weeks in mice, muscle stem cells proliferate actively and build up muscle tissue, after which muscle enters a steady-state where muscle mass increase occurs only in response to stress in form of injury or exercise. Muscle stem cells are activated upon cell stress to answer the demand created by injury-mediated tissue loss, but the cells only have a restricted regenerative potential, which gradually decreases with age.

3.1 Skeletal muscle stem cells and muscle differentiation

Satellite cells are tissue-resident stem cells of skeletal muscle that reside mitotically quiescent on top of myofibers, underneath the basal lamina. Satellite cells are typically found in large numbers in the vicinity of NMJs, at the ends of myofibers, and close to blood vessels. Satellite cells, and their progeny, are a heterogeneous group of cells in terms of the expression of muscle stem cell markers (such as Myf5, M-cadherin, integrin-α7, CD34), as individual cells express a different setup of molecular markers and also show variability in the expression levels of myogenic proteins (Sacco et al., 2008). The expression of the transcription factor Pax7 is, however, a ubiquitous and well-established marker for all quiescent and proliferating satellite cells in all muscle types across species (Seale et al., 2000). The molecular variability of satellite cells (within a muscle and between muscle types) is considered to stem from environmental factors that mold the satellite cell niche, generating differences in the differentiation potential of individual cells. Also the amount of satellite cells varies between muscle types. In addition to satellite cells, several other types of myogenic stem cells exist that can readily contribute to muscle differentiation and regeneration. Examples of such are the muscle resident PW1+/Pax7− interstitial cells (Mitchell et al., 2010) and pericytes (Dellavalle et al., 2007). Homing of bone marrow-originated MSCs to sites of muscle injury is also believed to contribute to the muscle regeneration process in vivo (Corbel et al., 2003). Nonetheless, the depletion of satellite cells severely blocks myogenic regeneration after muscle damage, and hence, the satellite cells are considered to be the most significant muscle stem cell population in acute muscle injury (Lepper et al., 2011). Researchers have shown that as few as seven still myofiber-associated satellite cells engrafted to dystrophic muscle can readily regenerate considerable parts of host muscle tissue (Collins et al., 2005), demonstrating the enormous myogenic potential of the cells. Satellite cells can, in suitable conditions, be differentiated to osteoblasts or adipocytes, suggesting that they ultimately have a mesenchymal stem cell character (Asakura et al., 2001).

When entering the cell cycle in response to a stress signal, the activated satellite cells immediately turn on the expression of muscle regulatory factors (MyoD, Myf5) (Cooper et al., 1999), and are thereafter considered to be part of the proliferative myogenic progeny of satellite cells, the myoblasts (illustrated in Figure 9). Myoblasts can either fuse to existing muscle fibers or fuse with other myoblasts to form new myofibers. The satellite cell pool is replenished by asymmetrical division, or alternatively, activated satellite cells can lose MyoD expression and return to a quiescent state (Pax7+, MyoD-) (Zammit et al., 2004). Myoblasts that enter the terminal myogenic differentiation program exit the cell cycle and upregulate the expression of transcription factors myogenin and Mrf4 (which are direct targets of MyoD and Myf5), thereby switching on an orchestrated series of events that involves increased motility and alignment of myoblasts preparing to fuse, as well as expression of muscle-specific proteins. The prerequisite for the initiation of differentiation is cell cycle inhibition (including upregulation of cell-cycle inhibitors) and repression of mitotic Cdk activity, whereas cell differentiation-related Cdks (such as Cdk5) are activated. Thus, the initiation of differentiation is balanced by (inhibitory) mitotic Cdks and (permissive) differentiation Cdks.

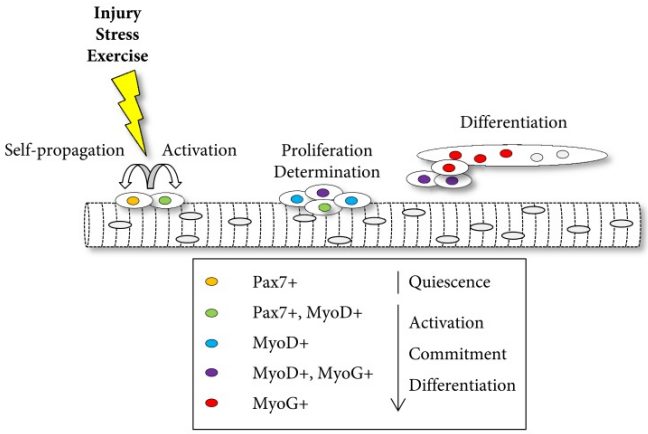

Figure 9 The expression of myogenic transcription factors during differentiation. Satellite cells are characterized by Pax7 expression, which is immediately lost upon commitment to differentiation. Pax7+, MyoD+ activated cells can also return to quiescence to maintain the stem cell pool. The molecular signature of proliferating myoblasts reflects their individual differentiation state. Eventually, at the stage of myotube formation, myogenin (MyoG) expression is downregulated from the newly formed myonuclei.

3.2 Muscle repair processes

Satellite cells can be activated by physical muscle damage (e.g. muscle injury, exercise, loss of innervation) or secreted factors (e.g. hormones, nutrients) to replenish muscle tissue. Importantly, a pool of satellite cells is always maintained in a quiescent state to ensure the preservation of the regenerative capacity of muscle, while other cells activate, proliferate and differentiate in response to muscle damage. The satellite cells become fewer with age and lose their myogenic potential due to the aging extracellular environment (Conboy et al., 2005), causing an age-dependent reduction in regenerative capacity of muscle (sarcopenia).

In practice, the process of muscle development, differentiation and healing all involve activation of satellite cells and their commitment into the myogenic differentiation program. Small injuries, like those that arise from exercise, mainly involve the activation of a few satellite cells that heal the microdamage of myofibers, whereas wounds of larger areas comprises the regeneration of several tissue types (myofibers, connective tissue, vasculature, nerves) and involves an inflammatory reaction. Destructive damage, disease conditions or excessive inflammation (myositis) may eventually lead to extensive myofiber death, sustained fibrosis and accumulation of adipocytes between myofibers, which impair normal muscle function and its contractile capacity. The injury-evoked inflammation is a crucial part of the healing process: macrophages and leukocytes, which are attracted to muscle in response to injury, are essential for phagocytosis of dead myofiber debris, but the inflammation also promotes activation of myogenic satellite cells and fibroblasts, which secrete transient (as well as fibrosis-related) extracellular matrix (reviewed in Mann et al., 2011; Yin et al., 2013; Chang and Rudnicki, 2014).

Muscle dystrophies derive from mutations of a variety of genes that mutually cause fragility in the muscle structure. Duchenne muscular dystrophy (DMD) is a recessive X-linked severe, but yet relatively common (1/3500 males), muscle wasting disease that derives from mutations in the muscle structural protein dystrophin. DMD causes severe progressive muscle wasting that will eventually cause premature death of the patient due to wasting of vital muscles, such as heart and those involved in breathing. The disruption of the dystrophin gene in mice (often referred to as mdx-mice) suitably recapitulates many DMD symptoms and is a commonly used mouse model in muscle wasting disease research. Another less common myopathy is Emery-Dreifuss muscular dystrophy, which is caused by lamina-disrupting mutations in genes that

encode lamin A/C or emerin, causing both skeletal and cardiac myopathy. The mechanisms of nuclear lamina-related muscular dystrophies are not well understood. Most myopathies progress slowly and do not affect the patient´s life expectancy, while others (such as DMD) dramatically shorten patient life-span. The differences in severity rise from different mechanisms, although the diseases have in common the continuous myofiber regeneration, fibrosis and adipocyte accumulation, as well as chronic inflammation of the muscle, which eventually depletes the myogenic stem cell pool, leaving the muscle incapable of repair. The progress of muscle dystrophy is a multi-cause process and the mechanisms of the cells involved are complex and poorly understood at a molecular level. On one hand, the muscle fibers are weak, while the myogenic cells lose their potential to self-renew or differentiate, which causes a gradual decline in muscle mass. Simultaneously, other cell types show an increased fibroblast and adipocyte differentiation potential, leading to the persistent problem of chronic fibrosis within muscle tissue. Muscle fibrosis is one of the key challenges in muscle dystrophies as it cannot currently be treated, or even restrained. At present, there is no treatment against muscle dystrophy, but especially muscle mass-supporting stem cell therapy has been successful in pre-clinical research models (reviewed in Mann et al., 2011; Miyagoe-Suzuki et al., 2012; Bareja and Billin, 2013).

Prolonged muscle damage initiated by secondary causes includes for instance sarcopenia, immobility-triggered atrophy, myositis and cancer- and AIDS cachexia. Although these muscle conditions can be more transient by nature, the muscle atrophy cannot be treated medically and thus they pose a serious healthcare problem due to their high prevalence. If curable, the recovery time from atrophy is usually long and has a huge impact on the quality of life of the patient.

3.3 Laboratory models in muscle research

Differentiation-competent immortalized muscle progenitor cell lines are frequently used to study muscle differentiation, one of them being the C2C12 cell line, which originates from a dystrophic mouse model (Yaffe and Saxel, 1977). C2C12 myoblasts differentiate promptly at high confluency and serum-withdrawal, after which they form multinucleated myotubes in 2D culture. Primary muscle progenitor cell cultures can be isolated from muscle by enzymatic digestion and physical disruption of muscle tissue, but have the disadvantage of the presence of other cell types in cultures: these cultures typically contain a combination of myoblasts, fibroblasts, adipocytes and other cell types, the proportions of which may vary inter-experimentally. Pure satellite cell cultures are commonly isolated with the help of nestin (Day et al., 2007) or Pax7-driven (Bosnakovski et al., 2008) fluorescent reporters, or by the selection of CD34+/integrin α7+/CD45-/CD31-/CD11b-/Sca1- cells by cell sorting (Pasut et al., 2012). A higher grade of purity can alternatively be reached by culturing of satellite cells from intact myofibers. These myoblast cell models, all of which have their own advantages and shortcomings, are useful to study myoblast proliferation and differentiation. There is, however, no in vitro model to study muscle regeneration, given the complex interplay between tissue types that occurs during muscle healing. Therefore, muscle injury is often studied in rodent injury models. The most commonly used injury types include chemical injury ($BaCl_2$, cardiotoxin), nerve injuries and physical injuries (cutting-, freeze-, or squeeze injury), all of which reflect slightly different injury conditions in terms of healing, inflammation and tissue remodeling.

AIMS OF THE STUDY

Prior to this work, there had been an interest towards the nestin/Cdk5/p35 signaling complex in the laboratory. First, Cdk5 was found to phosphorylate nestin during myogenic differentiation, leading to re-organization of nestin filaments (Sahlgren et al., 2003). Later, nestin was discovered to constitute a regulatory scaffold for the kinase in a model of stress-induced apoptosis in neuronal precursor cells (Sahlgren et al., 2006). This thesis aimed to question the role of nestin-Cdk5 interplay in muscle differentiation and healing. Considering the aforementioned work, the depletion of nestin was hypothesized to affect Cdk5 signaling also in myoblasts, with consequences on myoblast differentiation, muscle development and healing. Furthermore, nestin was hypothesized to retain its Cdk5-regulatory functions in cancer cell signaling, which was the research focus of the last part of this thesis. In total, this thesis consists of three studies that were designed as a natural continuum on the above-mentioned publications, and which together aimed to understand in detail the complex interplay between Cdk5 and the IFP nestin in development and disease. The specific objectives of the distinct projects were the following:

I. To understand the detailed role of the nestin scaffold in regulation of Cdk5 during muscle differentiation.

II. To assess in a broader perspective how skeletal muscle physiology and regeneration is affected by the genetic ablation of the nestin scaffold.

III. To examine the specific functions of Cdk5 in prostate cancer, and to address whether the concept of nestin/Cdk5 interplay could be extrapolated into a disease context.

EXPERIMENTAL PROCEDURES

Table 4 Methods utilized and reference to specific descriptions.

Method	Further references (in this thesis)
Cell culture	I, II, III
Cell sorting	III
Cdk5-kinase assay	II, III
Echo-MRI	II
Immunocytochemistry	I, II, III
Immunohistochemistry	II
Immunoprecipitation	I, II, III
LC-MS/MS	III
Live cell imaging	III
MTS-assay	I
Muscle injury	II
Myofiber cultures	II
Primary myoblast cultures	I, II
RT-qPCR	I, III
Statistical analysis	I, II, III
Transient transfections	I, III
Western blotting	I, II, III

1 Cell culture methods and experimental treatments

1.1 Cell lines

C2C12 myoblasts (I), HeLa cervical cancer cells (I), HEK293 epithelial cells (III), VCaP prostate cancer cells (III) and vimentin positive- and negative subclones of SW13 adenocarcinoma cells (I) were cultured in DMEM (Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS), 2 mM l-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. Subconfluent C2C12 myoblasts were induced to differentiate in DMEM containing 1% FCS with supplements. Myotube formation was followed as differentiation progressed (myotube formation occurred typically from 48 hours). LNCaP prostate cancer cells (III), PC-3 prostate cancer cells (III) and 22Rv1 prostate cancer cells (III) were cultured in RPMI-1640 media (Sigma-Aldrich) including supplements. Sodium pyruvate (Sigma-Aldrich) was added to LNCaP culture media to a final concentration of 1 mM. RWPE-1 prostate epithelial cells were cultured in keratinocyte serum free media (Life Technologies) with 5 ng/ml human recombinant EGF and 50 µg/ml bovine pituitary extract. LNCaP and 22Rv1 hormone depletion (-androgen) was conducted by exchanging medium to 10% charcoal/dextran treated FCS (Thermo Fisher Scientific) in phenol red-free RPMI-1640 (Gibco) with supplements. Hormone stimulation (+androgen) included the addition of 1 nM synthetic androgen mibolerone in the previous conditions. Hormone starvation/stimulation lasted 16-24 hours.

1.2 Experimental treatments

To study how the expression and solubility state of nestin affects p35 stability (I), HeLa cells and vimentin positive and negative SW13 subclones that were previously transfected (p35, Cdk5 and Nest-640 or GFP for HeLa and Nest-640 and p35 for SW13) were treated with 10 µM calcium ionophore A23187 (Calbiochem). A23187-treatment induces highly selective Ca^{2+} influx to cells, causing activation of the calcium-dependent calpain proteases and p35 cleavage. After controlling the viability of the cells by microscopy, samples were collected for Western blot analysis after 0, 2, 4 and 6 hours treatment. To address whether inhibition of PKCζ affects

nestin protein levels during differentiation (I), C2C12 myoblasts were induced to differentiate while treated with control peptide (Scr-P) or PKCζ-inhibitor (pseudosubstrate; PS) (MilleGen Biotech) at 20 μM concentrations (de Thonel et al., 2010). Samples were harvested for Western blotting after 48 and 72 hours, when prominent myotubes were apparent. Inhibition of Cdk5-activity with 10 μM roscovitine was compared to vehicle (dimethyl sulfoxide; DMSO) controls of similar concentrations in both C2C12 myoblasts (I) and prostate cancer cells (III). Fresh roscovitine was supplemented daily in differentiating myoblasts, and every two days in prostate cancer cells.

Neuregulin treatment for hyperactivation of ErbB-receptor/Akt kinase signaling in prostate cancer cells (III) was conducted through the addition of recombinant human neuregulin-1 (100 ng/ml) to growth media overnight (16h), after which samples were collected for Western blot. An equal concentration of PBS was used as control. To study protein stability in the absence of Cdk5 (III), protein synthesis was stalled with the translation-inhibitor cycloheximide (CHX) at 10 μg/ml. Ethanol was used as solvent to reconstitute CHX powder, and was used as control vehicle at comparable concentrations.

1.3 Transient transfections and plasmids

Before transfection through electroporation, HeLa and SW13 cells (I) were collected in Opti-MEM (Life Technologies). Cells were electroporated in the presence of DNA plasmids with a single electric pulse (220 V, 975 μF) in electroporation cuvettes using Bio-Rad Gene Pulser. Cells were then diluted in DMEM growth media and incubated 24-48 hours before further experiments. C2C12 (I) and LNCaP (III) cells were transfected with overexpression plasmids using JetPEI transfection reagent (PolyPlus Transfection) according to manufacturer´s protocol, and PC-3 cells with Lipofectamine LTX with PLUS reagent (Invitrogen) according to instructions of the manufacturer. Overexpression plasmids are listed in Table 5. Transient downregulation of Cdk5 (III) or nestin (I, unpublished) by siRNA in C2C12, LNCaP, PC-3, HEK293 or 22Rv1 cells was performed using Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to instructions of the manufacturer in antibiotic-free cell culture media. siRNA oligos were purchased from Qiagen (species specific GeneSolution for nestin or Cdk5 downregulation, as well as All Stars scrambled negative control) and used at a final concentration of 50 nM. All cells were incubated a minimum of 24 hours after transfection before further experiments.

Table 5 The origin of DNA constructs used in experiments.

DNA construct	Origin	Reference
CA-Akt-GFP	(Peuhu et al., 2010)	III
DN-Cdk5 (D145N)	Kind gift from Professor Harish Pant	I
Nest-640-GFP	(Sahlgren et al., 2006)	I
p35	Addgene	I, III
pcDNA	Kind gift from Professor Harish Pant	I, III
pEGFP	Clontech	I, III
WT-Cdk5	Kind gift from Professor Harish Pant	III

1.4 Cell proliferation assays

In the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay to assess proliferation (I), C2C12 cells were transfected with nestin or scrambled siRNA, re-suspended the next day and plated on 12-well plates in equal numbers. After 24 hours, a solution of MTS (Promega) and phenazine methosulfate (Sigma-Aldrich) was added to the cells, and the optical density was measured with Victor^2 plate reader (PerkinElmer) after one and two hour incubation. As MTS is converted to a colored formazan product in

metabolically active cells, colorimetric analysis of cell cultures can be utilized to study the metabolic state of cells, which is higher in faster proliferating cells.

To study cell population size in prostate cancer cells with and without Cdk5 (III), LNCaP and PC-3 cells were plated in equal amounts, transfected with siRNA, and counted every 24 hours with the trypan blue exclusion-technique in a Bürker cell counting chamber. VCaP cells were transfected similarly and counted after 0 and 72 hours. For double transfection, LNCaP or PC-3 cells were first transfected with overexpression plasmids overnight, and Scr or Cdk5 siRNA the following night, after which the cells were incubated for 48 hours and finally counted. Western blotting lysates were collected from all experiments to verify transfection efficiency.

For live cell imaging (III), LNCaP and 22Rv1 cells were plated, transfected overnight and then continuously imaged with a phase contrast Cell-IQ imaging platform (CM Technologies) with CO_2 supply at 37°C. Cell confluency of at least three separate areas was quantified using Cell-IQ Analyser-software (CM Technologies) with constant settings, and results plotted as relative area growth.

2 Mouse strains and tissue preparation

Nestin$^{-/-}$ and wild type mice (II) (Mohseni et al., 2011) were maintained in pathogen-free facilities at Central Animal Laboratory of Turku University in a 12:12h light-dark cycle and were provided with standard pelleted chow and tap water. Mice were sacrificed by CO_2 asphyxiation and cervical dislocation. Nestin knockout mice were in C57BL/6-background. Vimentin knockout and wild type mice (Colucci-Guyon et al., 1994) as well as FVB-n mice (I) were housed in a similar environment. For analysis of body composition (II), live animals were analyzed with EchoMRI body composition analyzer twice per measurement, of which the mean was calculated.

2.1 Primary muscle myoblast cultures

Primary mouse myoblasts from P3 FVB-n and vimentin knockout or wild type mice (I) were isolated from limb skeletal muscles. The muscle tissue was chopped and digested in 0.2% collagenase XI (Roche diagnostics) and 0.1% trypsin at 37°C for 60 minutes. Cells were filtered, centrifuged 1000 x g for 5 minutes and reconstituted in growth media (15% FCS, 2 mM l-glutamine, penicillin/streptomycin, 2.5 ng/ml fibroblast growth factor-β [Promega] in Hams-F10 [Sigma]) and plated on plastic. When cells reached 80% confluency, differentiation was induced by addition of 2% FCS/DMEM and supplements.

Primary mouse P3 myoblasts from nestin wild type and knockout mice (II) were isolated according to (Danoviz and Yablonka-Reuveni, 2012) from limb skeletal muscles with some modifications. Briefly, muscles were isolated, minced and digested in a solution of dispase II (StemCell Technologies) and collagenase A (Roche Diagnostics) for 60 minutes at 37°C. Myoblasts were released by serial trituration and filtration of the slurry. Cells were then centrifuged 1000 x g for 10 minutes and were reconstituted in growth media (20% fetal bovine serum, 10% horse serum, 1% chicken embryo extract in high glucose DMEM with standard supplements and 1 mM sodium pyruvate). Myoblasts were plated on gelatin-coated cell culture dishes or growth factor-reduced Matrigel-coated coverslips (BD Biosciences). Isolation of primary myoblasts from adult (2 month old) nestin$^{-/-}$ and wild type mice (II) from tibialis anterior (TA), extensor digitorum longus (EDL) and gastrocnemius muscles was executed according to Danoviz and Yablonka-Reuveni (2012) following the protocol described above. Differentiation of primary myoblast cultures was induced at 80% confluency by serum depletion (1% horse serum in DMEM including supplements).

2.2 Myofiber isolation

To study satellite cell function, live myofiber isolation (II) was performed according to (Pasut et al., 2012). Concisely, intact EDL muscles were isolated from adult mice and digested in 0.2% collagenase I (Calbiochem) solution for 60 minutes at 37°C, or until loose myofibers appeared from the muscle structure. The muscles were rinsed and serially titrated until more than 100 live myofibers were released from the muscle. Single myofibers were plated on Matrigel-coated dishes for attached cultures, horse serum-coated plastic for floating cultures or fixed immediately in 3% paraformaldehyde (PFA), and immunolabeled following the protocol for fixed cells.

2.3 TA-muscle injury

For assessment of muscle regeneration of nestin knockout mice (II), the mice were anesthetized with vaporized isoflurane. The left TA muscle was injured by half of its thickness by a transverse incision with a razor blade through the skin in the TA midpoint. The other TA muscle served as an internal control. The skin was sutured and 0.1 mg/kg intraperitoneal buprenorfine (Temgesic; Reckitt Benckiser Healthcare) (0 and 8 h post-operatively) and 5 mg/kg subcutaneous carprofen (Rimadyl; Pfizer Animal Health) (0, 24 and 48 h post-operatively) were used as analgesics. Animals were allowed to move freely in their cages and were housed with littermates until the time of sacrifice, after which muscles were collected and immediately fixed in 3% PFA.

3 Immunoprecipitation and cell fractionation

After collecting equal numbers of cells for immunoprecipitation, pellets were lysed 30 minutes on ice in lysis buffer. The following lysis buffers were used; for immunoprecipitation of nestin (I): 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.05% SDS, 5 mM EDTA, 5 mM EGTA, Complete Protease Inhibitor Cocktail (Roche Diagnostics); for immunoprecipitation of p35 (I), AR (III), Cdk5 (III) or Akt (III): 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, 5 mM EGTA including protease and phosphatase inhibitor cocktail. Samples were then centrifuged at 15000 × g for 10 min at 4°C. Supernatants were pre-cleared using protein A-Sepharose beads (Sigma-Aldrich) for at least 1 h at 4°C under rotation. 2 µg of each antibody was then added accordingly and incubated for 1-2 hours, after which protein A-sepharose beads were included in the mixture for further incubation. Last, the samples were washed three times with lysis buffer, re-suspended in Laemmli lysis buffer (LLB) and boiled. Immunoprecipitates were analyzed with Western blotting.

In cell fractionation assays (III), LNCaP cells were collected 48 hours after transfection. Cells were lysed in 10 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$, and 5 mM KCl with protease and phosphatase inhibitors for 30 minutes on ice, after which lysates were cleared with 720 × g centrifugation for 3 minutes. The cytosolic (supernatant) and membrane fractions (pellet) were collected after separation (20000 × g, 30 minutes), after which membrane fractions were washed once (20000 × g, 30 minutes). LLB was added to samples, and they were finally boiled and analyzed with Western blotting.

4 Western blotting

For Western blot analysis (I, II, III), cells were harvested in LLB and boiled. Lysates were resolved by sodium dodecylsulphate-polyacrylamid gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose (Protran, Sigma-Aldrich) (I) or polyvinyl difluoride (Millipore) (II,

III) membranes using the wet-tank transfer method (Hoefer). Membranes were blocked in 5% fat-free milk and incubated with primary and HRP-conjugated secondary antibodies (Table 6), and proteins were visualized using enhanced chemiluminiscence (GE Healthcare) on X-ray film (Fujifilm). Western blots in (I) were subjected to densitometric quantification using ImageJ software.

Table 6 List of antibodies used and their specific application.

Antibody	Manufacturer (clone/number)	Application	Reference
Actin	Sigma-Aldrich (AC-40)	WB	I, III
Akt	Cell Signaling (#9272)	WB, ICC	III
p-Akt Ser473	Cell Signaling (#9271)	WB	III
AR	Santa Cruz (N-20)	WB, ICC	III
p-AR Ser308	Santa Cruz (sc-26406-R)	ICC	III
β-catenin	BD Biosciences (14)	WB, ICC	III
Caspase-3	Cell Signaling (#9662)	WB	III
Cdk5	Thermo Scientific (DC34)	WB, IP	I, II, III
Cdk5	Santa Cruz (C-8)	WB, IP	II
Desmin	Santa Cruz (RD-301)	WB	I
Desmin	Cell signaling (#4024)	WB, ICC	II
GAPDH	Cell Signaling (#14C10)	WB	III
GFP	BD Clontech (JL-8)	WB, IP	I
p-GSK3β Ser9	Cell Signaling (#9336)	WB	III
Hsc70	StressGen (SPA-815)	WB	I, III
Ki-67	AbCam (15580)	WB	I
MHC	Santa Cruz (H-300)	WB	I
MyoD	Santa Cruz (C-20)	ICC	II
Myogenin	Santa Cruz (M-225)	WB	I
Nestin	BD Pharmingen (611659)	WB	I, II
Nestin	BD Pharmingen (556309), Rat 401	IP, ICC, IHC	I, II
Nestin	AbCam (10C2)	WB	unpublished
p-nestin Thr316	(Sahlgren et al., 2003)	WB	I
p21	Santa Cruz (C-19)	WB, ICC	I, III
p27	Santa Cruz (C-19)	WB	I, III
p35/p25	Santa Cruz (C-19)	WB, IP	I, II, III
PARP-1	Sigma-Aldrich (C-2-10)	WB	III
Pax7	DSHB	ICC	II
PCNA	Sigma-Aldrich (P-8825)	WB	I
S6	Cell Signaling (#2217)	WB	III
p-S6 Ser235/236	Cell Signaling (#4858)	WB	III
Troponin T	Sigma-Aldrich (JLT-12)	WB, ICC	I, II
Vimentin	BD Pharmingen (550513)	WB	I, II
Vimentin	Cell signaling (D21H3)	WB, ICC	II
Vimentin	Sigma-Aldrich (V9)	WB	I
Anti-rabbit HRP	Promega	WB secondary ab	I, II, III
Anti-rat HRP	GE Healthcare	WB secondary ab	I, II, III
Anti-mouse IgG HRP	GE Healthcare	WB secondary ab	I, II, III
Anti-mouse IgG1 HRP	Southern Biotechnology	WB secondary ab	I, II, III
Anti-mouse IgG2a HRP	Southern Biotechnology	WB secondary ab	I, II, III
Anti-mouse IgG2b HRP	Southern Biotechnology	WB secondary ab	I, II, III
Anti-mouse Alexa 488, 555, 633	Invitrogen	ICC secondary ab	I, II, III
Anti-rabbit Alexa 488, 546, 633	Invitrogen	ICC secondary ab	I, II, III

5 Immunocytochemistry and immunohistochemistry

For immunocytochemistry (ICC), cells were plated on coverslips and processed as described for each experiment (I, II, III). Briefly, cells were fixed with 3% PFA, rinsed in phosphate buffered saline (PBS) and permeabilized with 0.5% Triton X-100 for 5-10 minutes. Samples were then washed with PBS and blocked in 1-5% BSA for at least 60 minutes, after which samples were incubated in primary antibodies according to specific needs (I, II, III). After three washes,

samples were incubated in secondary species specific Alexa fluorescent probe-conjugated antibodies (Invitrogen), diluted 1:500-1:1000. All antibodies are presented in Table 6 and used as described in specific references (I, II, III). After four consecutive washes, cells were mounted in Vectashield with DAPI (Vector laboratories) (I), or ProLong Gold with DAPI (Life technologies) (II, III). Same protocol was used for immunolabeling of myofibers, where single myofibers were transferred from one solution to another. Confocal images were produced using Zeiss LSM 510 META and Zeiss LSM 780 laser scanning confocal microscopes at the Cell Imaging Core at Turku Centre for Biotechnology. Images were processed with ImageJ, Bioimage XD and Zen Software (Zeiss) software.

For immunohistochemistry (IHC) (II), muscle samples were collected and fixed in 3% PFA, dehydrated and embedded in paraffin. 2 µm thick sections were cut and collected on Superfrost plus slides (Thermo Scientific). Samples were stained with hematoxylin and eosin, or specific antibodies. IHC samples were imaged with Pannoramic 250 Slide Scanner (3DHISTECH) and analyzed with Case Viewer and Pannoramic Viewer software (3DHISTECH). The analysis of muscle fiber size and injured area was done manually using Pannoramic Viewer.

6 Flow cytometry

Transfected cells (III) were trypsinized, collected and re-suspended in propidium iodine buffer (40 mM Na-Citrate, 0.3% Triton X-100, 50 mg/ml propidium iodine [Sigma-Aldrich]) for 10 minutes at room temperature. Samples were then analyzed in duplicates with LSRII flow cytometer (BD Biosciences) with HTS platform at Turku Centre for Biotechnology. The fraction of nuclear fragmentation events (sub G1) was gated for as a measure of cell death.

7 Cdk5 kinase activity

Primary myoblasts from 3 months old male mice (II) were induced to differentiate for 24 h, after which the cell were collected in PBS, and the cell pellet was lysed for 30 minutes in lysis buffer on ice (50 mM Tris [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.05% SDS, 5 mM EDTA, 5 mM EGTA, Complete Protease Inhibitor Cocktail [Roche Diagnostics], PhosSTOP Phosphatase Inhibitor Cocktail [Roche Diagnostics]), and samples were centrifuged. Prostate cancer cells (III) were treated and lysed as above. The soluble fraction was pre-cleared with sepharose G beads (GE Healthcare) and incubated with Cdk5 antibody (DC34) for one hour. Protein G sepharose was added and samples were incubated under rotation for 2.5 hours. Samples were washed once with lysis buffer and two times with kinase reaction buffer (50 mM HEPES pH 7.2, 0.1 mM EDTA, 0.1 mM EGTA, and 5 mM $MgCl_2$). A mixture of ATP and 3 µCi radioactively labelled [γ-^{32}P] ATP was added to the beads to a final concentration of 100 µM. Histone H1 (Sigma-Aldrich) or AR were utilized as Cdk5 substrates. Kinase reaction took place at 30°C for 30 minutes, and was discontinued by the addition of LLB and boiling. The samples were run on 12.5% SDS-PAGE and stained with Coomassie brilliant blue. After drying, the ^{32}P-labelled substrates were visualized on X-ray film.

8 In vitro phosphorylation and mass spectrometry

For analysis of endogenous AR PTMs (III), androgen stimulated VCaP and calyculin A-treated (50 nM, 10 minutes) AR overexpressing HEK293 cells (Rytinki et al., 2012) were collected and lysed, after which AR was immunoprecipitated as described above in the presence of protease and phosphatase inhibitors (Roche). Immunoprecipitates were resolved on SDS-PAGE, gels were stained with Coomassie brilliant blue, and the appropriate bands were excised from the

gels. In-gel digestion with trypsin, phosphopeptide enrichment by TiO_2 affinity chromatography and analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS) were performed as described previously (Imanishi et al., 2007) with some modifications. For LC-MS/MS analysis of the digests with and without enrichment, an EASY-nLC II nanoflow liquid chromatograph coupled to LTQ Orbitrap Velos mass spectrometer and an EASY-nLC 1000 coupled to a Q Exactive mass spectrometer (Thermo Fisher Scientific) were used. Database search was performed against Swiss-Prot database (Homo sapiens) using Mascot 2.4.1 (Matrix Science) via Proteome Discoverer 1.4 (Thermo Fisher Scientific). Label-free quantification was performed using Progenesis LC-MS 4.1 (Nonlinear Dynamics).

For identification of in vitro Cdk5 phosphorylation sites on AR (III), AR was immunoprecipitated from VCaP cells as described before. Immunoprecipitates were then washed in kinase reaction buffer (10 mM HEPES [pH 7.2], 60 mM NaCl, 0.5 mM $CaCl_2$, 2.5 mM EGTA, and 2 mM $MgCl_2$). A mixture of ATP (Sigma-Aldrich) and 3 µCi [γ-^{32}P] ATP (final concentration 100 µM) and purified active Cdk5/p25-complex (a kind gift from Professor Sin-Ichi Hisanaga) was added to the beads. The kinase reaction took place at 30 °C for 30 min, after which LLB was added, samples boiled, and samples treated as described above for identification of endogenous AR PTMs by mass spectrometry.

For identification of in vivo Cdk5 phosphorylation sites on the AR (III), Cdk5 was downregulated with siRNA in LNCaP cells and incubated for 48 hours. Cells were then collected in ice-cold PBS and subjected to AR immunoprecipitation in the presence of phosphatase inhibitors as described previously, run on 7.5% SDS-PAGE and analyzed by LC-MS/MS as described for VCaP and HEK293 cells.

9 Statistical analysis

GraphPad Prism 5.0 statistics software was used to analyze statistical significance where needed (I, II, III) and analysis method was chosen according to experimental requirements (indicated in each experiment). $p<0.05$ was considered significant and marked with *; $p<0.01$ marked with **; $p<0.005$ marked with ***. Results are presented as mean±standard error of the mean (S.E.M.) and $n\geq 3$.

RESULTS AND DISCUSSION

1 Cdk5-nestin crosstalk adjusts myogenic differentiation (I)

Previous reports clearly indicated that nestin plays an important role in the regulation of the apoptotic functions of Cdk5 (Sahlgren et al., 2006). In addition, both proteins were found to be expressed in muscle (Sahlgren et al., 2003). At the time of this work, two other laboratories manifested an interest towards nestin-mediated scaffolding of Cdk5 at NMJs (Mohseni et al., 2011; Yang et al., 2011). In contrast to the neuromuscular models, the specific objective of this project was to answer whether nestin regulates the activity of Cdk5 to drive the pace of myogenic differentiation, given that nestin is prominent in mesenchymal stem cells and expressed in differentiating myotubes. Although the studied tissue types are the same, there is clear distinction in NMJ development and muscle differentiation both functionally and temporally. To address the differentiation signaling aspect, differentiating C2C12 mouse myoblasts were primarily utilized as a model system.

1.1 Nestin fine-tunes the pace of muscle differentiation

Nestin is dynamically expressed during myoblast differentiation, starting from the proliferating myoblasts. To address the specific role of nestin during myogenic differentiation, nestin protein was downregulated using RNA interference (RNAi) in proliferating C2C12 myoblasts, after which differentiation was induced by serum withdrawal. We observed that the absence of nestin caused an increase in the expression levels of early cell cycle-exit markers (p21, p27) and the myogenic transcription factor myogenin, and the cells also showed enhanced expression of muscle structural proteins (troponin t, myosin heavy chain; MHC), which are strongly induced upon initiation of differentiation (I: Figure 1). On the contrary, myoblast proliferation was not altered by the downregulation of nestin (I: Figure 1). The assessment of differentiation was executed both by Western blotting, as well as counting of MHC-positive differentiating C2C12 cells. Although efficient transfection was difficult to obtain in primary cells, nestin downregulation also promoted differentiation of freshly isolated myoblasts (I: Figure 3). While nestin downregulation seemed to enhance the differentiation pace, the reverse could be observed when nestin was overexpressed: the expression of differentiation markers was clearly suppressed after Nest-640 transfection compared to GFP control (I: Figure 2). As the true effect of nestin on differentiation may have been partly masked by the weak transfection efficiency of Nest-640 in the heterogeneous Western blot lysates, differentiating cells were quantified by immunolabeling of the differentiation markers MHC and troponin-t. The expression of the GFP-tagged Nest-640 was found to strongly delay (or nearly abolish) differentiation compared to GFP controls, when GFP/MHC and GFP/troponin t double positive cells were counted (I: Figure 2). Thus, when nestin is found in excess amounts in proliferating myoblasts, the process of differentiation is severely hampered.

Together these results suggest that nestin is not a passive bystander in muscle precursors, but that it is rather required for the fine adjustment of initiation of the differentiation process. Due to its expression in proliferative cells, nestin is often expected to affect cell division. Instead, we found that nestin acts on the cell cycle of myoblasts only after induction of differentiation, as its depletion causes premature differentiation after a stimulus, and as nestin siRNA did not affect myoblast proliferation. By standing at the gates of differentiation, nestin seems to regulate the earliest events of differentiation, the cell cycle exit, which is crucial for commitment of differentiation. The precocious cell cycle exit, as seen by increased cell cycle inhibitor

expression in nestin siRNA transfected myoblasts, also results in earlier expression of muscle structural proteins as the differentiation process as a whole is accelerated. These experiments demonstrated for the first time an unexpected role for nestin in cell differentiation, rather than proliferation. As nestin is widely and specifically expressed in mesenchymal stem cells in numerous tissues (Table 3), its role in initiation of differentiation of other cell types would be highly interesting to assess. Although the expression pattern of nestin during muscle differentiation is unique, suggesting it may play a role at several levels in this particular system, it is possible that the presence and balance of nestin in mesenchymal stem cells may determine differentiation of other cell lineages as well.

1.2 The importance of the nestin scaffold in regulation of p35 turnover in myoblasts

Cdk5 activity is known to be required for muscle cell differentiation (Lazaro et al., 1997). To establish a link between the observed nestin-associated effects on differentiation and Cdk5, the activity of Cdk5 was constrained by inhibition with roscovitine, as well as with the expression of DN-Cdk5, alongside nestin downregulation with specific siRNA. While nestin knockdown cultures differentiated at a faster pace than Scr control cells according to expectations, the abrogation of Cdk5 activity with either method effectively counteracted the hastening effect of nestin ablation (I: Figure 5), suggesting that the proteins do in fact act in the same signaling pathway. Thus, similarly to that which Sahlgren and colleagues (2006) showed to occur in dying neuronal progenitor cells, the depletion of nestin seemed to cause an increase in Cdk5 activity, which in the muscle context would translate into a faster differentiation pace. To provide verification for this hypothesis, the protein levels of Cdk5 activators were analyzed in nestin-modulated myoblasts. Supportive to our hypothesis, downregulation of nestin did increase the cleavage of p35 to the more stable p25 (I: Figure 6), a process known from previous reports to be required for C2C12 differentiation and generation of adequate Cdk5 activity (de Thonel et al., 2010). Overexpression of Nest-640 did in turn decrease p25 generation at the whole population level, again a process presumably masked by the low transfection efficacy (I: Figure 6). At the single cell level, the hampered p25 generation would be expected to have strong inhibitory effects on the initiation of the differentiation program. Unfortunately, the absence of suitable tools, such as antibodies that would allow the assessment of p35 kinetics through microscopy at the single cell level, debilitates the detailed examination of the exact signaling events. Our results, together with previously published data did, however, support that nestin regulates p35/p25 turnover in differentiating myoblasts, and consequently Cdk5 activation at the right time, and in the correct place within the cell.

Nestin is a minor IFP in muscle in terms of protein abundance and requires a polymerization partner to form filaments. It is known from before that nestin exists in both soluble and filamentous pools, the stoichiometry of which changes dynamically during myoblast differentiation due to phosphorylation (Sahlgren et al., 2003). Thus, we asked ourselves whether the solubilization of nestin has actual consequences for p35 signaling. To address this issue, we utilized vimentin positive (v+) and negative (v-) subclones of SW13 adenocarcinoma cells. As the SW13 cells do not express other IFPs that would permit nestin filament formation, Nest-640 remains completely soluble in v- cells, while it incorporates at least partially into normal filaments in v+ cells. Interestingly, co-transfection of Nest-640 and p35, and the following p35 co-immunoprecipitation from both v+ and v- cell lines, demonstrated that soluble nestin (in v- cells) enhances Cdk5/p35 complex formation (I: Figure 6). The association of Cdk5 and p35 driven by soluble nestin also has a stabilizing effect on p35 after calpain activation with A23187 (I: Figure 6), suggesting that when Nest-640 is transfected into myoblasts (or HeLa cells [I: Figure 6]), p35 is stabilized by the soluble Nest-640 particles. This nestin-mediated sequestration of p35 is likely to perturb the initiation of myoblast differentiation. When p35

cannot be cleaved to p25, differentiation does not occur. Similarly, Cdk5/p35 complex formation has been reported to decrease p25 generation in neurons (Kamei et al., 2007). Whether the effect of nestin occurs at the level of Cdk5 activation or re-localization (both of which are affected by p25 generation [Patrick et al., 1998, Asada et al., 2008]) cannot be answered at this moment, as not even the myogenic substrates of the kinase are understood.

These results did highlight the important effect of nestin solubility on Cdk5 activity; a feature of IFs often overlooked. The PTMs of IFs not only affect the localization of the proteins, but usually promote filament disassembly (Yamaguchi et al., 2005). We found that in the case of nestin, its solubilization has a function in Cdk5-mediated scaffolding, and it seems to act to re-direct kinase activity or suppress hyperactivation of the kinase to prevent unwanted activities that may be harmful for the differentiation process. Thus, when nestin is targeted with siRNA in myoblasts, also the soluble pool of nestin is depleted, leading to uncontrolled cleavage of p25 and stimulation of differentiation. Similar features of other IFPs have been described; not only in relation to phosphorylation, which has been under intense investigation, but also other factors regulate the solubility and signaling properties of IFs. For example, the regulation of lamin solubility and its displacement to the nucleoplasm by LAP2α is a decisive factor in myoblast cell cycle exit (Gotic et al., 2010). Recently there has been a great interest to understand how substrate stiffness regulates (at least partly through phosphorylation) the assembly of both cytoplasmic and nuclear IFs (Murray et al., 2014; Swift et al., 2013) with prominent consequences on cell fate. Thus, the solubility of IFPs has become a widespread interest in the research community.

Another intriguing detail is the nature of the filament structures that nestin forms. As described in the review of literature, soluble nestin-vimentin differentially regulates IDE activity (Chou et al., 2009), not only stressing the importance of nestin solubility in regulation of IDE enzymatic activity, but also underscoring the need of an appropriate polymerization partner even in a non-filamentous state. On the other hand, also vimentin expression and turnover is regulated by nestin, at least under particular circumstances (Chou et al., 2003; Eliasson et al., 1999). With the help of primary myoblasts isolated from vimentin null mice, we found that the ablation of vimentin did cause an increase in the differentiation pace, presumably through alteration of nestin dynamics during differentiation (I: Figure 4). Although nestin protein levels were not greatly decreased in vimentin$^{-/-}$ myoblasts, the filament properties of nestin could be dramatically different, even if nestin looks similar in WT and vimentin$^{-/-}$ myoblasts under the microscope, at least at later stages of differentiation when desmin expression has been induced (Figure 10). Whether nestin forms filaments with vimentin or desmin, is probably a matter of temporal regulation, as vimentin is substituted by desmin during differentiation. Preferentiality of nestin polymerization partners in muscle has not been addressed. In general, the knockout of desmin has little effect on the appearance of nestin (Carlsson et al., 1999), highlighting the dynamic compensation of individual IFs by other family members.

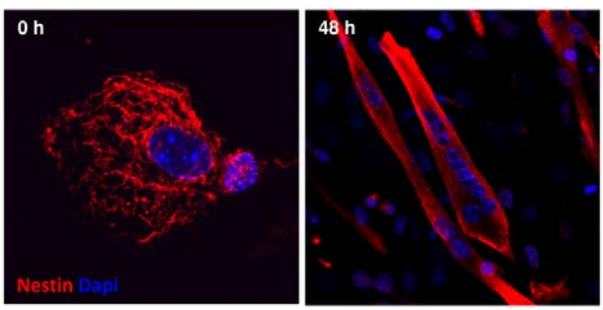

Figure 10 Nestin in proliferating and differentiating primary vimentin$^{-/-}$ myoblasts. Initially, when proliferating vimentin null myoblasts have low expression levels of desmin, the nestin cytoskeleton forms truncated filaments (left panel). In differentiated vimentin knockout myotubes, where desmin levels are generally high (48 h), the nestin cytoskeleton forms long filaments that follow the shape of the myotubes, similar to vimentin WT myotubes (not shown).

In terms of cytoskeletal regulation of Cdk5, it is

tempting to speculate that other IF substrates (such as lamins or neurofilaments) may constitute a similar scaffold for the kinase that impact cell fate determination. Similarly to IFs, also actin seems to form a regulatory scaffold for Cdk5/p39; if actin polymerization is disturbed by cytochalasin D treatment, p39 is dramatically reorganized and Cdk5 activity increased (Humbert et al., 2000). Although the mechanism is very different, other cytoskeletal elements play a crucial role in correct Cdk5 regulation, especially in neurons, but perhaps also in other model systems.

1.3 Cdk5 talks back: self-regulation through nestin

Intriguingly, a further level of complexity of the regulatory function of the nestin scaffold system is added by Cdk5 itself. It is known that the Cdk5-mediated phosphorylation of nestin promotes solubilization of the filament structure (Sahlgren et al., 2003; Sahlgren et al., 2006). If Cdk5 activity is inhibited in differentiating myoblasts, phosphorylated nestin (p-Thr316) is diminished together with the soluble nestin pool (Sahlgren et al., 2003). As soluble nestin attracts Cdk5/p35 complex formation to protect p35 from calpain-induced deregulation, it seems that Cdk5 can modify its own activation through acting on the solubility state of nestin. This hypothesis is difficult to study in differentiating myoblasts due to the presence of many simultaneous IFPs that change dynamically during the process, all of them affecting the nestin cytoskeleton in their own way. It is however important to note, that the levels of soluble and phosphorylated nestin are elevated during differentiation (Sahlgren et al., 2003), suggesting that restriction of Cdk5 activity in an auto-regulatory feedback-loop might be required at later stages of differentiation. During myotube formation, the nestin cytoskeleton is actively re-modelled, also including an increase in nestin protein levels before it is abolished completely from mature muscle. Interestingly, if Cdk5 activity is inhibited directly (with roscovitine or DN-Cdk5), or indirectly through PKCζ (with PS peptide inhibitor), nestin levels are held low, as differentiation is not initiated at all, and the characteristic up-regulation of nestin protein levels during differentiation does not occur (I: Figure 7). It is possible, that the up-regulation of nestin during differentiation (which is initiated by Cdk5 itself) is required for temporally appropriate regulation of Cdk5 activity, and the nestin cytoskeleton may serve different purposes during different time points of the differentiation process.

Thus, the nestin-mediated scaffolding of Cdk5 during muscle differentiation closely resembles its cytoprotective function during neuronal apoptosis (Sahlgren et al., 2006): when nestin is depleted, p35 is uncontrollably processed to p25, leading to hyperactivation of Cdk5 with the outcome of increased cell death or increased myogenic differentiation (Figure 11). In the NMJ system, the role of nestin in regulation of Cdk5 is slightly different due to intrinsic distinctions between the systems. For example, p25 is not known to have a function at the NMJs. Whereas nestin siRNA increases p25 cleavage in differentiating myoblasts (a process associated with Cdk5 hyperactivation), nestin depletion (or mutation of the Cdk5-phosphosite) decreases both membrane bound and total Cdk5 activity at NMJs (Yang et al., 2011),

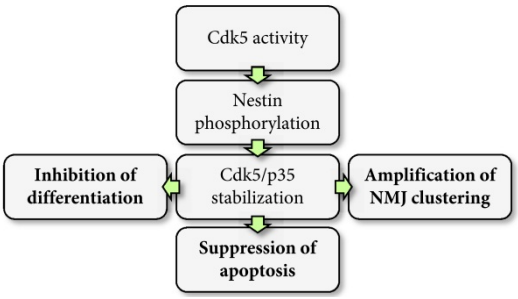

Figure 11 Cdk5-nestin crosstalk determines muscle differentiation, NMJ clustering and neuronal apoptosis. Cdk5 regulates the turnover of its own scaffold through phosphorylation, which consequently enhances Cdk5/p35 complexing, at the expense of p25 generation during differentiation and apoptosis. At the NMJ, only p35 has been found to be involved in Cdk5 activation. Depending on the model system, the promotion of Cdk5/p35 has different effects on the cellular outcome.

which eventually prevents NMJ dispersion. In other words, (phosphorylated) nestin is first required for activation of Cdk5/p35 at NMJs. Activated Cdk5 phosphorylates and increases soluble nestin at NMJs (Yang et al., 2011), leading to further Cdk5/p35-complex formation at the membrane. Thus, in both models soluble nestin causes Cdk5/p35 stabilization. At NMJs, soluble nestin/Cdk5/p35 is required for normal NMJ development, and depletion of nestin would disturb Cdk5 activation. On the other hand, we know that calpain-mediated p25 generation is required for muscle differentiation (de Thonel et al., 2010). When (hypothetically soluble) nestin is depleted from differentiating myoblasts, p35 is uncontrollably cleaved by calpains, causing an increase in Cdk5/p25 generation, which promotes differentiation. When nestin is overexpressed using the Nest-640 construct, the balance of nestin filaments is severely hampered. A noticeable fraction of the overexpressed Nest-640 remains soluble (I: Supplementary figure 2), and it can be hypothesized, that this particular fraction severely interferes with proper Cdk5 activator turnover. However, further experiments using phospho-deficient nestin-mutants would be a good addition to pinpoint the exact role of Cdk5-mediated phosphorylation in its auto-regulation during muscle differentiation. Thus, the activity of Cdk5 is dynamically regulated by the kinase itself, through nestin, in a complex auto-regulatory feedback loop.

This work demonstrated for the first time the involvement of nestin in regulation of a differentiation process. Through intricate means, nestin and Cdk5 were found to regulate each other in a highly balanced system, which is prone to signaling inputs in a dynamic environment. Similar IFP-kinase interactions have been described in various contexts, highlighting that the IFPs, their interactions as well as their often unnoticed assembly state, are important fine-tuners of signaling processes that determine cellular fate. Through its actions on Cdk5 and its activators, nestin negatively controls the cell cycle exit (or perhaps promotes a less differentiated state) after a permissive differentiation stimulus has been granted. Besides Cdk5, it is equally plausible that the functions nestin reach to yet unidentified proteins. Also, whether nestin acts at the doorways of differentiation in other cellular systems, needs to be addressed in future experiments.

2 Nestin regulates skeletal muscle homeostasis and regeneration in vivo (II)

While the previous study focused on the molecular signaling functions of the nestin scaffold with regards to Cdk5 regulation mainly in C2C12 myoblasts, the aim of the following study was to address how the genetic knockout of the nestin gene affects skeletal muscle, and therefore, body homeostasis. Whereas the nestin deficient mouse was described to have a NMJ organization defect, the authors did not observe other prevalent phenotypes or dysfunctions (Mohseni et al., 2011). In addition, skeletal muscle as such was described as having no phenotype under normal conditions. As many IFP knockout models appear normal until they are challenged, our main aim was to study skeletal muscle under stress in mice lacking nestin.

2.1 Gender and age-dependent variability in myoblast p35 expression

Data from myoblasts (I) indicated that the transient downregulation of nestin in C2C12 myoblasts increased the processing of p35 to p25 upon serum withdrawal, thereby accelerating the progress of differentiation. Therefore, primary myoblast cultures were initially isolated from WT and nestin deficient mice, and differentiated in low-serum media to examine their behavior in relation to previous results. Contrary to our expectations, there was no significant change in expression of differentiation markers between WT and nestin null myoblasts (II: Figure 1), and myoblasts from both genotypes seemed to form myotubes well in culture. Hence, differentiation seemed to occur regardless of the nestin status of the cells. We could not detect

any differences in the organization or protein levels of other major IFPs in nestin null myoblasts that would suggest direct compensation of the lack of nestin (II: Figure 1). Interestingly, a similar null phenotype of differentiating myoblasts has been observed with other IFP knockout model systems: when for example desmin, the major IFP in muscle, is genetically targeted, desmin$^{-/-}$ myoblasts seem to form myotubes at comparable rates to their wildtype counterparts (Li et al., 1997; Milner et al., 1996). On the contrary, when desmin is transiently downregulated in C2C12 cells, a clear defect in myoblast differentiation is discerned already at early stages (Li et al., 1994), pointing out that there may be some unidentified acquired compensatory mechanism in the desmin null mice that allow cell functioning in a situation that would otherwise result in excruciating chaos. Thus, it is feasible that a comparable situation occurs in nestin knockout myoblasts, explaining the difference of transient nestin downregulation compared to genetic targeting. It should also be taken into consideration that there may be molecular differences in the immortalized C2C12 cells compared to primary myoblasts that may affect the expected outcome. C2C12 cells do, however, have a similar setup and dynamics of the major IFPs, as well as Cdk5-related proteins, making them a convenient model system to study the function of nestin.

Although the rate of differentiation was similar in all studied myoblast cultures, which were derived from mice of different age and gender, p35 protein levels were found slightly elevated in nestin knockout myoblasts from young (P3) males, and the difference was even more striking in adult (2 month old) male-derived myoblast cultures (Figure 12). Even though p35 levels were highly upregulated in male nestin null myoblasts, p25 was never found to be altered. As p25-content is the key switch to allow Cdk5 activation and differentiation (de Thonel et al., 2010), it is perhaps not completely surprising that no change in differentiation could be distinguished either. Unexpectedly, female myoblasts never showed genotype-derived changes in p35 protein levels (Figure 12). The mechanism of p35 accumulation in nestin null myoblasts is not clear, but is not likely to be a direct consequence of nestin ablation, the effect being both gender- and age specific. To more directly analyze the impact of increased p35 on Cdk5 activity in nestin$^{-/-}$ myoblasts, Cdk5 was immunoprecipitated from differentiating myoblasts from adult male mice, and subjected to kinase activity measurements. As a result of increased p35 expression, total Cdk5 kinase activity towards histone was found to be dramatically increased in nestin null myoblasts after 24 hours of differentiation (II: Figure 1), a relatively early time point of differentiation when Cdk5 activity is known to increase strongly.

It can only be speculated at this time that the highly active Cdk5 in nestin knockout myoblasts is sequestered by p35 mainly to the membrane and cytoplasm. As nestin null myoblasts showed no increased p25 generation during any time points after induction of differentiation, the overly active kinase would not perform its differentiation-related functions any differently from the wildtype situation, and hence, no change in the differentiation pace could be distinguished. Regardless of the mechanism utilized to target nestin (by siRNA in I, or genetically in II), which seem to differently affect p35 turnover, the absence of the nestin scaffold in myogenic tissue causes deregulation of Cdk5 activity in both cases. To fully understand the myogenic signaling of Cdk5, it would be essential to analyze the substrates of the kinase in muscle, and to understand completely the distinction in substrate specificity between p35 and p25, i.e. why p25 generation is essential in the first place. After all, the kinase is likely to have differentiation-related substrates, which do not relate to its functions at the NMJ at all, as evidence supports that Cdk5 abrogation has direct effects on the expression of early myogenic transcription factors and somitogenesis (Philpott et al., 1997). Furthermore, it would be highly interesting to study more specifically Cdk5 activity levels in other tissues in the nestin null mice. Such dramatic changes in kinase activity, as observed in the myoblasts, could be detrimental under particular circumstances, such as apoptosis or during tissue-specific stress (e.g. in kidneys), or

alternatively, in Cdk5-related disease contexts (e.g. diabetes or cancer). As p35 levels did not reveal great variation in muscle lysates, it is probable that a specific stimulus (such as myoblast activation in this case, or a stress situation in other organs) may be required to distinguish altered Cdk5 signaling and related phenotypes in the nestin null mouse.

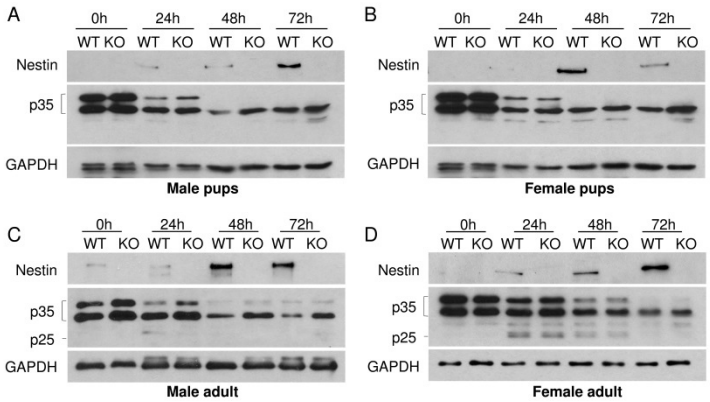

Figure 12 Expression of p35 in proliferating and differentiating primary nestin$^{-/-}$ myoblasts. Myoblast cultures were isolated from mice of different age and gender and induced to differentiate, after which samples were analyzed for p35 expression using Western blotting. p35 levels did not differ greatly in male (A) or female (B) pup (P3) myoblast cultures, except slight elevations of p35 in knockout (KO) male pups compared to wildtype (WT). p25 is not visible due to short exposure of the film. On the contrary, nestin null male adult myoblasts showed great up-regulation of p35 in all time points analyzed when compared to WT samples (C), whereas this trend was never observed in females (D). p25 generation can be faintly detected at 24 hours in both males and females, but never showed genotype-dependent differences. The p35 double band likely represents differentially modified PTM isoforms.

2.2 Nestin$^{-/-}$ mice have less lean mass and show signs of spontaneous muscle regeneration

Regardless of the unexpected myoblast data, we wanted to make a comprehensive analysis of nestin$^{-/-}$ mice with emphasis on skeletal muscle, and a series of follow-up experiments were designed to cover the remaining questions regarding muscle health and stem cell function. First, the weight of male mice was measured from the age of 1 month onwards. During the first time point (age 4 weeks), the nestin$^{-/-}$ mice were indistinguishable from the control animals, but from the time they reached sexual maturity (circa 8 weeks onwards), nestin deficient mice started to appear smaller in terms of total body weight. At the age of 3 months, the weight difference was small, but recurring (less than one gram, statistically significant) (II: Figure 2). At an old age (>15 months) nestin$^{-/-}$ male mice weighed significantly less (divergence more than 7 grams by average) (II: Supplementary figure 1), including some remarkably lean individuals. To understand the basis of the observed differences, mice of different age and gender were examined with EchoMRI body composition analyzer for lean and fat mass distribution. While the mean fat mass was not significantly different, nestin$^{-/-}$ male mice did show significant reductions in lean mass at the age of 3 months and in the age group >15 months (II: Figure 2, Supplementary figure 1). The body weight and body composition analysis revealed a similar trend for 12 week old females (II: Supplementary figure 1), demonstrating the gender-independent nature of the lean nestin$^{-/-}$ phenotype. As an interesting note, the lesser body weight of nestin$^{-/-}$ mice seems to stem nearly directly from the reduced amount of lean mass, at least when genotypic mean values are compared.

To further dissect the cause of the lean mass phenotype, individual skeletal muscles from the back limbs of mature male mice were weighed, some of which were found to be significantly lighter in weight. Both the TA (mainly a fast twitch muscle) and EDL (fast twitch) muscles

weighed significantly less in nestin$^{-/-}$ mice, whereas the slow soleus showed no marked weight alterations between genotypes (II: Figure 3). TA and soleus are typical weight bearing muscles. As the size of individual myofibers was similar in WT and nestin null mice (II: Figure 3), the myofibers do seem to mature normally, and hence, a defect maturation is not a likely explanation for the difference in muscle weight. Furthermore, the amount of Pax7+ satellite cells in nestin knockout muscle was comparable to the WT (II: Figure 1), suggesting that the lean muscles of mice lacking nestin are not a consequence of satellite cell deficiency either. When examining the detailed histology of nestin null TA, EDL and soleus muscles, we were greatly surprised to find an increased incidence of myofibers containing centrally located nuclei in TA and soleus muscles (II: Figure 4), indicating that there is a spontaneous degeneration/regeneration cycle in nestin deficient muscle. A mild regenerative phenotype was not observed by Mohseni and co-authors (2011), possibly due to the fact that the muscle phenotype varies with muscle types, and can therefore be easily missed. Furthermore, it was obvious that the severity of the muscle regeneration/degeneration phenotype varied between individuals, as it was found to be more severe in certain animals, and more evident in particular muscles. We could not detect other signs of severe muscle malfunction, such as intramuscular fat accumulation or spontaneous fibrosis, which can be typically found in more difficult muscle conditions (II: Figure 4).

Thus, in contrast to what was previously reported, we found that nestin$^{-/-}$ mice do show a reduction in muscle mass and have a mild spontaneous regenerative skeletal muscle phenotype. The cause of the mild regenerative phenotype observed in nestin null mice remains to be identified, but seems to stem from the properties of the mature myofibers. As we could not observe differences in satellite cell numbers, and as the differentiation as measured by myoblast commitment and in 2D-cultures was apparently normal, the regeneration does not seem to stem from aberrant myoblast behavior (II: Figure 1). Given that nestin null muscle develops normally (Mohseni et al., 2011), and that any differences in body weight seems to come with age (and does not originate from differences in myofiber size per se), it seems as myofibers indeed develop and mature normally. Based on these observations, it seems particularly clear that the observed lean muscles and the increased number of regenerating myofibers in nestin knockout mice derive from myofiber-intrinsic defects that are likely to accumulate during minor muscle stress, such as exercise. As innervation of muscle is a major pathway that determines muscle growth, it is possible that the defective NMJs of nestin knockout mice (Mohseni et al., 2011) may contribute to the observed phenotype. On the other hand, the regenerating fibers were often dispersed evenly throughout the muscle, suggesting that the phenotype is not caused by impaired neuronal input. As nestin is found in large quantities at myotendinous junctions as well, it is possible that it may play a role in the mechanosensing properties of the muscle, which is only revealed when the muscle is under stress. Therefore, it is possible that muscle lacking nestin is more fragile to tissue stress, which is the case of many other IFPs. However, these questions need to be addressed more carefully with further experimental data.

2.3 Muscle healing after injury is delayed in nestin$^{-/-}$ mice

Finally, to study the functionality of nestin deficient muscle during ultimate stress, a muscle injury model was established. In brief, muscle injury was induced under anesthesia by a transverse cut in the TA muscle and the mice were allowed to recover freely motile in their cages until the time of sacrifice. Based on immunohistochemical staining of nestin during different time points of recovery after injury, nestin was first upregulated in proliferating myoblasts in the site of injury after 2-3 days. Later, when regenerating myofibers started to dominate the wound area (after one week), nestin was detectable in the newly forming

myotubes, and could be detected until day 28 after injury (II: Figure 5). Given the long-lasting expression of nestin in our model system, day 28 was finally selected as the end-point to study whether there is a difference in muscle healing between genotypes. The degree of regenerating muscle was determined by the area occupied by regenerating myofibers (identified by centrally located myonuclei) compared to the total muscle area, and was found to be greatly increased in nestin null muscle, suggesting that nestin is required for proper muscle healing (II: Figure 5). The muscle scar area was histologically indistinguishable between genotypes, suggesting that also the impaired regeneration stems from a myofiber-intrinsic defect (II: Figure 5), and is likely to originate from same causes as the spontaneous regeneration. We could not observe histological changes in the early time points in regenerating muscle between WT and nestin null muscle (Figure 13), which suggests that myofiber degeneration and differentiation occurs at similar times in both genotypes.

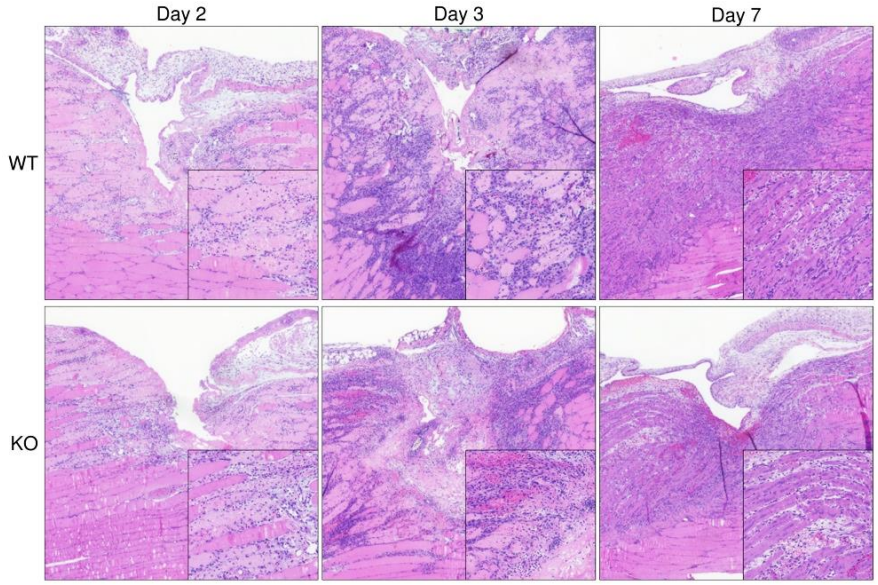

Figure 13 Early stages of muscle regeneration in WT and nestin null muscle. The TA muscle of WT and nestin null (KO) muscle was subjected to a cut injury, and the injury was allowed to heal for indicated time points. Samples were collected for H&E staining. Both genotypes showed similar pattern of early regeneration at a histological level, i.e. death and degeneration of injured myofibers, which appear light and fragmented under the microscope (day 2), immune cell infiltration (particularly evident at day 3), and finally, myotube formation through myoblast fusion (day 7). Large images are 2 x 2 mm, and inserts 0.5 x 0.5 mm in size.

Together these results highlighted that nestin is required for maintenance of normal muscle homeostasis, and that its absence delays muscle healing. Although the phenotype is not severe, the results do indicate that nestin is an IFP that will affect muscle condition and health. The genetic ablation of other IFPs has revealed their functions in many aspects of muscle biology. Desmin seems to be a major IFP required for myoblast commitment, but it is also an active organizer of muscle architecture (Agbulut et al., 2001; Li et al., 1994; Li et al., 1997; Milner et al., 1996). Recent studies of two separate synemin knockout mouse models revealed that this IFP is involved in maintenance of membrane integrity and muscle structure (Garcia-Pelagio et al., 2015; Zhenlin et al., 2014). Also keratin 19 seems to act as a structural component of muscle (Stone et al., 2007). IFs are also essential parts of the cell adhesion structures that maintain tissue integrity during stress; a fundamental function of IFs which will inevitably affect tissue stress tolerance. Therefore, it seems as the IFs are especially important for skeletal muscle integrity, probably by acting as organizing building blocks of this highly stress-prone organ, at

least partly through their scaffolding functions exerted towards signaling proteins. In this sense, the functions of nestin that have portrayed it as a signaling scaffold for Cdk5 during differentiation, as well as its role in maintaining mature and regenerating muscle (perhaps through Cdk5-dependent mechanisms as well), suit well in the proposed functions of other IFs in skeletal muscle. However, all IFPs seem to have highly specialized and unique functions, as demonstrated by the various muscle phenotypes of IFP knockout animals.

3 Cdk5 controls prostate cancer cell proliferation through Akt and AR (III)

Although prostate cancer genomics is complicated by the heterogeneity of the tumors, it can be roughly stated that some of the most common genetic lesions in aggressive prostate cancer are found in *AR*, *TP53*, *PTEN*, and *SPOP* (a subunit of a E3 ubiquitin ligase), and *RB1*, but also include gene fusions between *TMPRSS2* and the Ets family of transcription factors (e.g. TMRPSS2-ERG fusion products) (Barbieri et al., 2012; Taylor et al., 2010). Most therapeutic approaches against prostate cancer are targeted towards the AR, but the tolerance against these drugs in CRPC calls for the need of novel strategies. There is, therefore, a great need to understand prostate cancer development and progression in terms of gene alterations and signaling pathways that contribute to cellular oncogenic behavior.

3.1 Nestin - a Cdk5 scaffold in prostate cancer?

Prior to initiation of this work, there were indications pointing towards seemingly separate roles for both Cdk5 and nestin in prostate cancer cell motility (Kleeberger et al., 2007; Strock et al., 2006), but only limited information existed on the role of the proteins in prostate cancer proliferation. Since then, several publications have added to the knowledge of both Cdk5 and nestin in cancer, however leaving often considerable gaps regarding their specific signaling functions. Interestingly, both nestin (Reimer et al., 2009) and Cdk5 (Kino et al., 2007; Kino et al., 2010) have been separately implicated in regulation of members of the steroid hormone receptor-family: the Cdk5-mediated N-terminal phosphorylation of GR (Ser203, Ser211) was associated with suppression of receptor activation through altered GR/co-activator interactions (Kino et al., 2007). Thus, Cdk5 was found to affect the specificity of GR targets, thereby fine-tuning its signaling capacity. Phosphorylation of GR Ser203 and Ser266, detected with phospho-specific antibodies, was also increased in vimentin-positive subclones of glioma cells (where nestin remains filamentous) compared to subclones lacking vimentin (soluble nestin) (Reimer et al., 2009). As manipulation of the vimentin cytoskeleton has direct consequences on nestin solubility and stability, the Cdk5-related scaffolding properties of nestin (if relevant) would be altered in this model system. Given the strong link between nestin and Cdk5 in muscle and neuronal models (Sahlgren et al., 2003; Sahlgren et al., 2006), it is inviting to raise the question whether the nestin-dependent GR regulation could involve a Cdk5-dependent mechanism. With this hypothesis in mind, I speculated further whether a similar connection could exist in prostate cancer, where the steroid hormone receptor AR is one of the key drivers of prostate carcinogenesis. Steroid hormone receptor PTMs are known to play a crucial role in receptor transactivation, localization, stabilization and target gene selection. Thus, the first purpose of this project was to assess whether Cdk5 could regulate AR together with (or independently of) nestin in prostate cancer cells.

The possible role of nestin as a Cdk5 scaffold in prostate cancer is an obvious question with regards to the theme of this thesis. However, a few key observations suggest that nestin-mediated scaffolding of Cdk5 is not unconditionally crucial for Cdk5 functions in prostate cancer. Firstly, nestin is not very abundantly expressed in prostate cancer cells and is found prominently in filamentous organization only in PC-3 cells, and at lesser extent in DU-145

prostate cancer cells (Kleeberger et al., 2007). 22Rv1 prostate cancer cells do express nestin, but its organization is atypical and appears dotted under the microscope. In patient samples, nestin was found to be expressed in the cancer cells of high grade metastatic lesions as well as the primary tumors themselves (Kleeberger et al., 2007), and the authors argued that nestin expression correlates with advanced disease. On the contrary, all tumor cells in the prostate cancer samples in the Human Protein Atlas-web resource (Uhlén et al., 2005) appear nestin negative after analysis with four different antibodies, whereas the tumor stroma showed nestin immunoreactivity (www.proteinatlas.org; accessed 12.5.2015). Therefore, nestin expression in prostate tumor cells in situ remains unclear. Kleeberger and co-authors (2007) also reported that nestin mRNA is faintly induced by androgen deprivation in LNCaP prostate cancer cells, whereas long term androgen starvation failed to induce nestin at protein levels in our hands (J. Lindqvist, J. Eriksson, unpublished data). As nestin is not found in all cell lines (i.e. LNCaP) where Cdk5 in turn is found, the proteins are likely to be regulated by separate means. In addition, Cdk5 does not seem to affect nestin protein levels or organization significantly in PC-3 cells, nor has an interaction been observed between the two proteins (J. Lindqvist, J. Eriksson, unpublished data). Therefore, the nestin scaffold does not seem to be essential for Cdk5 functions in prostate cancer cells.

3.2 Cdk5 phosphorylates AR and guides its target gene specificity

Regardless of the less convincing nestin-Cdk5 connection in prostate cancer, experiments focusing solely on Cdk5 were pursued, as Cdk5 has several phosphorylation motifs on AR, including serines 81, 94, 256, 308, 424 and 524 in the AR N-terminal domain, and Ser650 in the ligand-binding domain (Chen et al., 2006). Between the N-terminal domain and ligand binding domain lays the AR DNA-binding domain, essential for AR-mediated transcriptional regulation. The AR is activated in a multistep process through ligand binding (i.e. by testosterone, 5α-dihydrotestosterone or similar compounds), which causes dimerization and nuclear translocation of the receptor complex. In the nucleus, AR binds to androgen response elements in the DNA, thereby modulating transcription of its target genes. Additionally, AR activation is regulated by molecular interactions of co-factors that bind to the activation function 1-domain (spanning amino acids 101-370 in the N-terminal domain). Thus, the AR N-terminal domain is crucial for maximal AR activity, and so it eventually fine-tunes the androgen response (reviewed by Helsen and Claessens, 2014).

To investigate potential Cdk5 phosphorylation sites on AR, sensitive mass spectrometry was chosen as a screening method. AR was immunoprecipitated from VCaP cells, phosphorylated in vitro with recombinant Cdk5/p25-complex, and analyzed by mass spectrometry (III: Figure 2). Compared to non-phosphorylated samples (-kinase) Ser308 was the only identified phosphorylation site induced by Cdk5/p25, and counteracted by roscovitine. To confirm the phosphorylation of Ser308 in vivo, LNCaP cells were subjected to Cdk5 or control siRNA, after which AR was immunoprecipitated and analyzed for altered phosphosites, revealing that Ser308 phosphorylation was indeed suppressed in cells lacking Cdk5 (III: Table 1, Supplementary figure 3). The presence of p-Ser308 in LNCaP cells grown in regular growth media was confirmed by immunolabeling (III: Figure 2), and the staining pattern was found to partly overlap with Cdk5. While this set of experiments demonstrated that Cdk5 phosphorylates AR Ser308 both in vitro and in vivo, only little is known of the biological significance of Ser308 phosphorylation. The only recognized kinase for Ser308 to date is Cdk11p58/cyclin D3 (Zong et al., 2007). Contrary to other Cdks in prostate cancer, Cdk11p58/cyclin D3-activity has a negative impact on androgen-dependent proliferation and AR activity measured by luciferase reporter (Zong et al., 2007). Furthermore, the effect of the kinase on AR activation was shown to depend on Ser308, since S308A mutation counteracted the outcome of the kinase on AR

activity (Zong et al., 2007). However, the evidence does not directly support that Ser308 phosphorylation would block AR transactivation, as a phospho-mimicking mutant was never studied and because the phospho-deficient mutant did not increase AR activity further. It is possible, that a yet unidentified mechanism initiated by Ser308 could play a role in AR regulation. As an interesting note, S308A mutation causes the abrogation of two metabolically labelled phosphopeptides in the phosphopeptide mapping-experiments that originally identified this particular phosphosite in vivo (Gioeli et al., 2002), opening the possibility that Ser308 could certainly be regulating a sequential cascade of other PTMs. Thus, the role of Ser308 phosphorylation should be investigated in more detail, especially as it lies in the regulatory activation function-1 domain that coordinates AR activity and interactions.

While this project was ongoing, a separate report from another laboratory suggested, after phosphoantibody-analysis, that Cdk5 promotes AR Ser81 phosphorylation (Hsu et al., 2011). Ser81 phosphorylation is important mainly for AR stability, and the site is also phosphorylated by other Cdk family members (Cdk1 and Cdk9) (Chen et al., 2006; Chen et al., 2012b). In reality, it is likely that several kinases (Cdk1, Cdk5, Cdk9) contribute to the maintenance of the exceptionally high phosphorylation stoichiometry of Ser81 (Gioeli et al., 2002), a phosphorylation site crucial for receptor stability (Chen et al., 2006), localization and chromatin binding (Chen et al., 2012b). Our results added to the knowledge by demonstrating a second Cdk5 phosphorylation site, Ser308, on AR. In the in vivo context, Cdk5 can almost certainly phosphorylate several AR residues (both Ser81 and Ser308) depending on the cellular circumstances, thereby initiating a unique PTM-fingerprint that has implications for AR functions. By analyzing a broad range of known AR targets, we found that in the absence of Cdk5, some AR target genes were suppressed, while others were induced or unchanged, highlighting that Cdk5 plays a complex role in AR target gene specification (III: Figure 2, Supplementary figure 4). In contrast to previous publications (Hsu et al., 2011), our results imply that Cdk5 can also have adverse effect on specific AR targets, which resembles the Cdk5-mediated regulation of GR (Kino et al., 2007). It is possible, that in the absence of Cdk5, AR co-activator or co-repressor complexes do not form normally, thus giving rise to the altered gene selectivity of the AR, which has consequences on the proliferative androgen pathway.

3.3 Cdk5 regulates prostate cancer cell proliferation

To study the function of Cdk5 in prostate cancer, a number of different cell lines were initially utilized to provide a comprehensive view of Cdk5 functions. In Table 7, where the molecular signature of the used cell lines is summarized, the term androgen response refers to the capacity of androgens to stimulate cell proliferation. In 22Rv1 prostate cancer cells, androgen stimulation does induce AR stabilization and activation, but does not increase their proliferation: in this case, the term “androgen responsive” is applied. Although present in most cells, the AR is altered in all utilized cell lines. A mutation in the LNCaP AR ligand binding domain causes both promiscuous activation by other steroid hormones and anti-androgens, as well as activation and nuclear localization of AR in the absence of ligands (Veldscholte et al., 1990). VCaP cells on the other hand, express as much as 12 times more AR than LNCaP cells, which makes the cells extremely sensitive to androgens (Waltering et al., 2009). The cell lines are also different in terms of p53 status. Thus, the cell lines have distinct molecular characteristics that reflect different stages of prostate carcinogenesis, and these distinctions were taken into consideration when interpreting experimental results.

To study the proliferation of prostate cancer cells, Cdk5 was downregulated using RNAi, and cells were counted or followed with live cell microscopy. The absence of Cdk5 was found to dramatically abrogate the proliferation of LNCaP, 22Rv1 and PC-3 cells (III: Figure 1, Supplementary figure 1, Supplementary videos 1-8), while VCaP was the only cancer cell line

not to respond to Cdk5 modulation (III: Figure 6). Also, overexpression of WT-Cdk5 increased cell population size in LNCaPs (III: Figure 1), suggesting that Cdk5 can boost proliferation, at least to a certain extent, in the cancer cells that from the beginning have a high metabolism. The cells did not show signs of increased apoptosis. When taking into consideration the specific molecular characteristics of the used cell lines, two key features could be distinguished. Firstly, the impact of Cdk5 siRNA on the proliferation of the AR-null PC-3 cell line was not less effective than its effect on the AR-positive LNCaP and 22Rv1. Thus, it seemed as the AR status of the cells did not matter for the effect of Cdk5 on proliferation. This was rather surprising, since we and others found that Cdk5 does regulate AR through phosphorylation, which is one of the major growth-promoting pathways in prostate cancer. Next, the intact Phosphatase and tensin homolog (PTEN) status and the virtually non-existent Akt activity of VCaP cells at basal levels (Ha et al., 2011) seemed particularly eye-catching. Genetic aberrations that cause hyperactivation of the PI3K-Akt pathway in prostate cancer also include PIK3CA-mutations in addition to PTEN, and 1/3 of advanced prostate cancers have mutation in either one (Sun et al., 2009). Although 22Rv1 cells have normal PTEN, they are characterized by an activating mutation in PIK3CA encoding the catalytic p85α-subunit of PI3K; a mutation that leaves the Akt kinase hyperactive. Thus, the fact that the only Cdk5 siRNA non-responsive cancer cell line was independent of Akt, suggested that this signaling pathway should be examined more closely in relation to Cdk5.

Table 7 Molecular signature of utilized prostate cancer cell lines.

	LNCaP	VCaP	22Rv1	PC-3
AR status	positive (mutated)	positive (amplified)	positive (mutated)	null
Androgen response	androgen dependent	androgen dependent	androgen responsive	androgen independent
p53 status	WT	mutated	mutated	null
PTEN status	mutated	WT	WT	null
Origin	lymph node metastasis	vertebral metastasis	carcinoma xenograft	bone metastasis

3.4 Complex Cdk5-Akt-androgen interplay in prostate cancer cells

LNCaP cells were utilized as the main model system to study the signaling pathways downstream of Cdk5 due to their wide expression of signaling proteins, as opposed to other cell lines. Cdk5 was downregulated in LNCaP cells that were either depleted from all hormones or stimulated with synthetic androgen, as LNCaP cell proliferation is under strict regulation of the androgen receptor. In the absence of Cdk5, active (phosphorylated) Akt (Ser473) was strongly repressed (III: Figure 3). Similarly, several of its downstream targets were suppressed/activated accordingly. The Cdk5-dependent regulation of Akt targets was, however, found to be variably androgen dependent; while p-Akt and p-GSK3β (Ser9; indicating activation of the kinase) levels were equally low after Cdk5 siRNA in both androgen starved and androgen stimulated conditions, phosphorylation of ribosomal protein S6 (p-S6, Ser235/236) was highly androgen dependent. Androgen treatment caused by itself a stabilization of S6 and an increase in its phosphorylation, denoting hormone dependent activation of the mTOR-S6 kinase-S6 growth-promoting signaling pathway. It seems as in the presence of hormones, the inhibitory effect of Cdk5 downregulation is actively counteracted by simultaneous AR feedback into the strongly growth-promoting mTOR pathway. Although the growth-promoting pathways regulated by Cdk5 can translate differently, depending on the specific protein expression pattern of the cell lines used, the effect of Cdk5 on p-Akt is androgen independent, which is particularly obvious

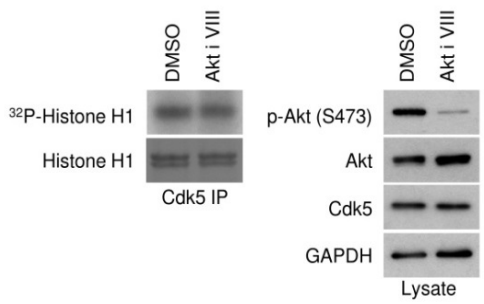

Figure 14 Effect of Akt inhibitor VIII on Cdk5 activity. Akt kinase activity was inhibited in LNCaP cells using 1μM Akt inhibitor VIII (Calbiochem) for 24 hours. Akt inhibitor VIII binds to the PH-domain of Akt, and therefore disturbs the membrane binding and the phosphorylation-mediated activation of the kinase. After treatment, Cdk5 was immunoprecipitated from DMSO and Akt inhibitor-treated samples (Akt i VIII) and subjected to kinase activity measurements in vitro using histone H1 as substrate and [γ-^{32}P] ATP incorporation as a measure of Cdk5 activity. Cdk5 activity was not affected by Akt inhibition.

in the AR null PC-3 cell line (III: Figure 3), and true for those prostate cancer cells that possess hyperactive Akt. Last, the relationship between Cdk5 and the multipurpose kinase Akt was further questioned through measuring Cdk5 activity after Akt inhibition, results which revealed that abrogation of Akt does not affect Cdk5 activity (Figure 14). Therefore Cdk5 can be placed upstream of Akt in the signaling hierarchy of prostate cancer cells, and not vice versa.

To understand better the mechanism of Cdk5-mediated Akt regulation, the intracellular localization of Akt was studied. When Akt is activated, it associates with phosphatidylinositols in the plasma membrane, where the kinase is activated through phosphorylation on serines 308 and 473. Therefore, the membrane localization of Akt is crucial for its activation. Using subcellular fractionation and imaging, we found that the membrane localization of Akt was indeed disturbed when Cdk5 was silenced (III: Figure 4). Furthermore, Cdk5 and Akt could be co-immunoprecipitated from LNCaP cells, suggesting that there is a direct interaction between the two proteins (III: Figure 4). Considering these results, Cdk5 seems to act as a crucial regulator of Akt activity in prostate cancer cells: without Cdk5, Akt activation at the membrane does not occur, and the growth-promoting effects of Akt are strongly suppressed. The Cdk5-Akt regulatory axis was further validated with rescue experiments, where expression of constitutively active (myristoylated) Akt partially rescued the growth retardation of Cdk5 downregulated LNCaP cells (III: Figure 6). Although the same experimental setup could not be repeated in the transfection-sensitive PC-3 cells, the roscovitine-induced lag in proliferation of PC-3 cells could be overcome when CA-Akt was expressed (Figure 15). Thus, regardless of the AR status of the cell, the effect of Cdk5 dysfunction in cell proliferation can be rescued by restoration of membrane bound, constitutively active Akt.

The results propose that Cdk5 is a key regulator of prostate cancer proliferation through its multimodal and simultaneous actions on both AR and Akt signaling. Although the Akt-pathway was ultimately the prime target of Cdk5-induced prostate cancer proliferation, the Cdk5-dependent regulation of AR is likely to be important in those cells, where AR is present. As many of the AR target genes that were found to be regulated by Cdk5 in an androgen-dependent manner are central cancer-associated proteins (III: Figure 2), this mode of regulation should not be underestimated. Especially in vivo, individual patients or even one single tumor will contain cells that differ in their proteome, and that rely on different mechanisms for survival. In this setting, Cdk5 will theoretically have the capability to act in several cell types in a pro-proliferative manner.

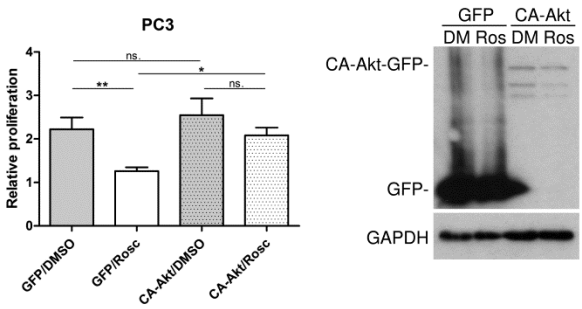

Figure 15 Expression of constitutively active Akt rescues roscovitine-induced lag in PC-3 cell proliferation. Cells were transfected with GFP or GFP-conjugated CA-Akt, re-plated the following day in equal numbers and immediately treated with DMSO or roscovitine. Cell numbers were counted after 48 hours. Relative proliferation indicates cell counts compared to the 0 hour time point. Results are plotted as mean±S.E.M (One-way ANOVA). Western blotting verifies efficient transfection.

Given the strong effects of Cdk5 inhibition on cell proliferation, we further questioned what effect Cdk5 downregulation would have on the cell cycle. In fact, the many growth-promoting and anti-apoptotic key signaling pathways in carcinogenesis are significantly intertwined. Akt is known to negatively regulate p53 stability through the direct phosphorylation of the p53 E3 ubiquitin ligase Mdm2 (Zhou et al., 2001). Therefore, the state of the p53-p21 signaling axis was assessed. As could be expected from the repressed Akt signaling, the downregulation of Cdk5 strongly upregulated the cell cycle inhibitors p53 (at protein level) and p21 (at protein and mRNA level) (III: Figure 5), which is reflected also in the strong cell cycle arrest of LNCaP cells in response to Cdk5 siRNA. Interestingly, p21 mRNA is also influenced by the androgen status: the Cdk5 siRNA-mediated cell cycle suppression is slightly alleviated in hormone-stimulated cells that are primed to proliferate faster, once again illustrating the complex relationship between the signaling pathways in the prostate cancer cell cycle. Thus, the lack of Cdk5 seemed to induce a p21-dependent cell cycle arrest, which is reflected in the altered cell proliferation. As an important note, the p53-p21 signaling is not a general Cdk5-regulated pathway, as PC-3 cells, which are equally Cdk5-dependent in terms of proliferation, are p53 null. Rather, the p53-p21 signaling provides yet another mode of action for the inhibited Akt and AR pathways in LNCaP cells to efficiently disturb proliferation. Especially when Cdk5 was inhibited with 10 μM roscovitine (III: Supplementary figure 2), which will target all cells in a population, compared to transfections that are effective only on a subpopulation, the growth-inhibitory effect of roscovitine was much stronger in LNCaP cells compared to PC-3. Therefore, it seems as LNCaP cells are more sensitive to Cdk5 inhibition, perhaps through the tripartite actions on these three imperative cancer-sustaining pathways. It is attractive to speculate that when Cdk5 is efficiently inhibited in LNCaP cells, its simultaneous effects on both Akt (including the strong p53/p21 upregulation) and AR, which the cells are in general highly dependent on, makes the cells especially sensitive to Cdk5 inhibition, with drastic consequences on proliferation. The androgen independent PC-3 cells, on the other hand, show similar degree of proliferation inhibition after both Cdk5 siRNA (III: Figure 1) and 10 μM roscovitine treatment (III: Supplementary figure 2). Importantly, titration of roscovitine concentrations demonstrated its efficiency already at lower concentrations than what was used in the original study (2.5–5 μM), when other Cdks would remain virtually unaffected (Figure 16).

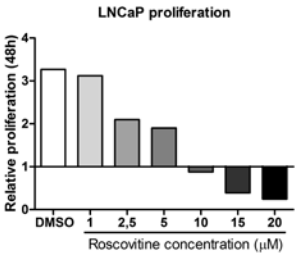

Figure 16 Effect of roscovitine on LNCaP population size. Cells were plated in equal amounts, treated with DMSO or various concentrations of roscovitine, and counted after 48 hours. Cell population size is displayed as relative growth (compared to the 0 h time point). At concentrations 10 μM roscovitine and higher, the cell population shrinks compared to the 0h time point.

Although many facts suggested that nestin is not required for Cdk5 functions in prostate cancer, nestin was found to regulate the same targets as Cdk5 in 22Rv1 cells: when nestin was downregulated with siRNA, both p-Akt and AR levels were altered (Figure 17). Since nestin depletion was found to cause an increase in Cdk5-activity in neuronal (Sahlgren et al., 2006) and muscle cells (I), it is tempting to speculate that a similar chain of events may occur in prostate cancer cells. If the hypothesis held, and the nestin-Cdk5 connection acted in a similar manner in 22Rv1 cells as in the other tissues, an increase in p-Akt and AR levels could be expected in nestin knockdown cells, due to increased Cdk5 activity, as indeed was the case (Figure 17). Thus far, the nestin-Cdk5 connection has not been verified in 22Rv1 cells and the observed phenomena of similar targets may be unrelated, but it would be an interesting aspect to address in more detail in future experiments. LNCaP cells are, in turn, naturally nestin deficient, and express no vimentin either that would allow nestin filament formation to occur. Therefore, with the lack of convenient prostate cancers model systems, the concept of nestin-Cdk5-AR interplay was not developed further. Instead of prostate cancer models, it could be more meaningful to study a possible nestin-AR interaction in a tissue like skeletal muscle, where both are present and have physiological functions. Also other model systems resembling prostate cancer, such as breast cancer models, would be interesting to approach in terms of Cdk5-nestin-mediated regulation of the hormonal signaling pathways. For example, breast cancer has many similarities to prostate cancer, where deregulated receptor tyrosine kinases are frequent, but where tumorigenesis is instead primarily driven by the estrogen receptor instead of androgen receptor. Nestin expression does in fact seem to correlate with poor prognosis in breast cancer (Gao et al., 2014; Piras et al., 2011). Given the emerging role of Cdk5 in breast cancer epithelial-mesenchymal transition (Liang et al., 2013), and our newly found role of Cdk5 as a regulator of Akt-signaling (which is commonly deregulated in breast carcinoma), it would be of great interest to study the Cdk5/nestin signaling interactions in breast cancer models in the future.

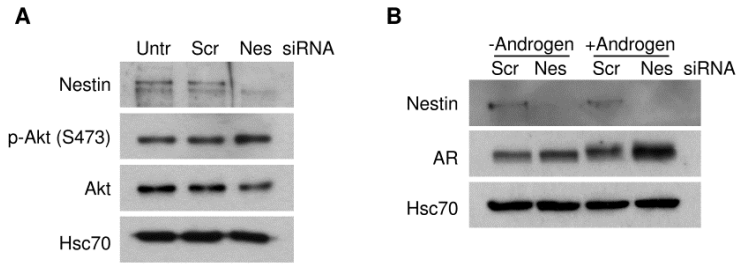

Figure 17 Nestin downregulation affects Akt and AR expression in 22Rv1 prostate cancer cells. (A) 22Rv1 cells were transfected with scrambled (Scr) or nestin siRNA (Nes), or left untreated (Untr), and samples were collected for Western blotting 48 hours later. Compared to controls, nestin knockdown increased the p-Akt/Akt ratio. (B) 22Rv1 cells were first transfected with indicated siRNA, and then either androgen treated or starved for 24 hours. Nestin downregulation did cause an increase in AR protein levels regardless of hormone treatment. Androgen treatment stabilized the AR and caused a PTM-induced band shift, as expected.

In the end, this work demonstrated that can Cdk5 act either separately, or simultaneously, on two important prostate cancer promoters, namely Akt and AR (Figure 18). It is intriguing to speculate that Cdk5 may offer a growth advantage for prostate cancer cells through promoting cell cycle progression. Cdk5 could even, under specific circumstances, compensate functionally for cell cycle Cdks in a cancerous environment, as the kinases have many mutual targets. Its multifaceted functions in neuronal cell death and nociceptive signaling have raised a therapeutic interest towards Cdk5 as a potential drug target in the field of neuropsychiatric diseases, analgesia and addiction. Given its well-reported role in cancer cell invasion and now proliferation, Cdk5 should be considered as a potential cancer drug target. Whereas Cdk5

inhibition in neuronal tissues may be beneficial in terms of development of novel pain relief medication and in the prevention of neuronal loss triggered by neurodegenerative diseases or ischemia, contraindications that may disturb the positive activity of Cdk5 in memory formation, podocyte biology or muscle differentiation are serious health risks that should be taken into consideration. However, roscovitine and well as other pan-Cdk inhibitors have generally been well tolerated in clinical trials, with minimal reported toxicity. This supports the fact that targeting of Cdk5 even with less-specific inhibitors or through other mechanisms could be a real opportunity in the drug development field.

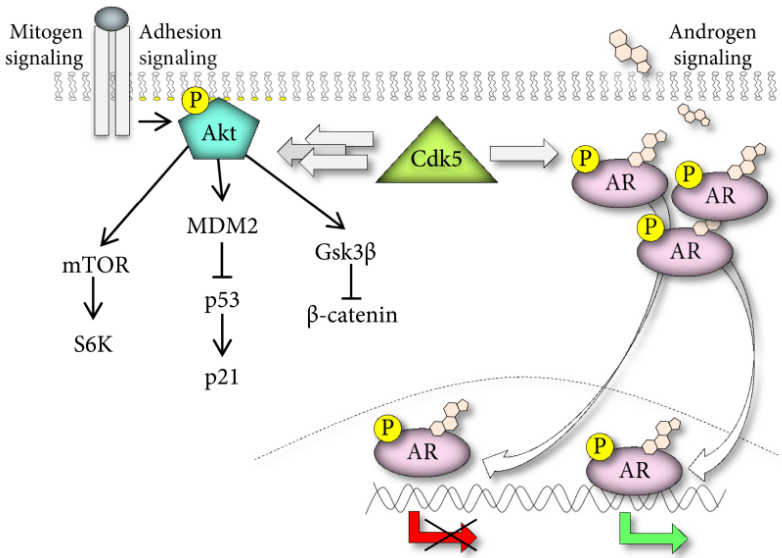

Figure 18 Cdk5 regulates prostate cancer proliferation through Akt and AR signaling. Cdk5 promotes the proliferation of prostate cancer cells through various mechanisms; firstly, Cdk5 phosphorylates the AR, which has target-gene selective effects on AR activation, and promotes prostate carcinogenesis through the androgen pathway. However, the androgen signaling axis is not fully required for Cdk5-mediated regulation of prostate cancer proliferation in an androgen independent stage. Instead, Cdk5 seems to mainly drive the proliferation of cancer cells through the multifunctional growth-promoting Akt kinase in both androgen dependent and independent cells. When Cdk5 is depleted from prostate cancer cells, Akt activation at the membrane is disturbed, and both pro- and anti-proliferative effectors of Akt are affected.

CONCLUDING REMARKS

During the progress of this thesis work, there has been an apparent growing interest towards understanding the non-neuronal functions of Cdk5 in the research community. Not only is Cdk5 considered to be a stress-sensor that determines the balance between life and death of a neuron, but it has newly found functions in cell transcription, proliferation, differentiation, cell architecture and motility in a wide range of dividing and terminally differentiated cell types, and in physiology and disease. This thesis studied the functions of Cdk5 in two atypical settings: muscle and proliferating cancer cells. By utilizing in vitro and in vivo methods, nestin was revealed to act as a critical scaffold for Cdk5 in muscle, as was originally hypothesized. The presence and state of the nestin scaffold ultimately determines whether Cdk5 is allowed to execute its p25-dependent functions following a differentiation-permissive signal, or alternatively, whether the myogenic cells continue proliferation and expansion instead. In cancer cells, the frequently differentiation-associated Cdk5 was found to promote proliferation instead, indications of which have existed in literature before. When unleashed, Cdk5 activity has the capability to boost many essential cell cycle-related signaling pathways. Thus, cancer cells seem to be able to utilize Cdk5 to gain a growth advantage compared to non-cancer cells, which in the case of prostate cancer, was found to be realized through its simultaneous actions on Akt and AR. Therefore, Cdk5 not only promotes initiation of differentiation processes, as noted already in early experiments, but the kinase can also advance cell proliferation in a disease context (Figure 19). Contrary to expectations, nestin did not appear to be a pre-requisite for the functions of Cdk5 in prostate cancer, although its modulation may affect the signaling capacity of the kinase in certain cell models.

Also the field of IFs is changing, as we are coming to understand the complexity of the phenotypes of the many knockout mouse models that were originally defined as "normal". An example was described yet again using the nestin knockout mouse: although appearing grossly normal, closer inspection of skeletal muscles of nestin null mice revealed underlying complications in skeletal muscle homeostasis and healing. Therefore, the results suggest that nestin plays a novel role in maintenance of muscle integrity. Although IFs play a significant role in the preservation of tissue integrity, studies on knockout and knock-in models of a variety of IF proteins are collectively suggesting that their function not only occur at the cellular level, but reaches from homeostasis of the whole body to regulation of metabolism, and ultimately disease. In intricate and unexpected ways they suppress, activate and modify cell signaling pathways through modulation of protein kinases and other signaling proteins, with broad-ranging consequences on tissue function and well-being.

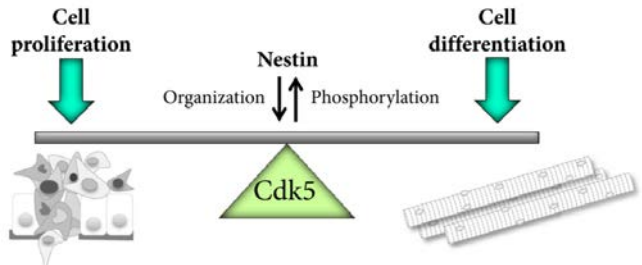

Figure 19 Cdk5 balances between cell proliferation and differentiation. Cdk5 has multiple newly found functions in determination of cell fate: Cdk5 activity promotes the progress of differentiation processes in neurons and muscle, whereas deregulated Cdk5 activity in cancer can support cell division instead. The signaling capacity of Cdk5 can be modified by the bi-directional relationship with the nestin scaffold, which fine-tunes the processes that Cdk5 is involved in.

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