CELLULAR RESPONSES TO STRESS AND KINASE SIGNALING ACTIVATION: APOPTOSIS AND DIFFERENTIATION

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Al mio papa´

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications and manuscript which are referred to in the text by their Roman numerals. In addition unpublished results are included.

I. Ferraris SE, Isoniemi K, Torvaldson E, Anckar J, Westermarck J, Eriksson JE. 2012. Nucleolar AATF regulates c-Jun-mediated apoptosis. Mol. Biol. Cell. 23(21):4323-32.

II. Ferraris SE, Anckar J, Eriksson JE. Nucleolar AATF promotes c-Jun activation in a JNK-independent manner. *Manuscript*

III. de Thonel A*, Ferraris SE*, Pallari HM, Imanishi SY, Kochin V, Hosokawa T, Hisanaga S, Sahlgren C, Eriksson JE. 2010. Protein kinase C zeta regulates Cdk5/ p25 signaling during myogenesis. Mol. Biol. Cell. 21(8):1423-34

* Equal contribution

ABBREVIATIONS

AATF Anti apoptosis transcription factor
Aβ β-amyloid peptide
AP-1 Activator protein 1
APAF1 Apoptotic protease activating factor 1
ATM Ataxia telangiectasia mutated
Bcl B-cell-lymphoma
BSA Bovine serum albumin
CAD Caspase activated DNAse
CARD Caspase recruitment domain
Cdk Cyclin-dependent kinase
CNS central nervous system
CRE cAMP responsive element
DAG diacylglycerol
DED Death effector domain
DISC Death inducing signaling complex
DR Death Receptor
DTT Dithiothreitol
EGF Epidermal growth factor
ER endoplasmic reticulum
ERK Extracellular signal-regulated kinase
FADD Fas-associated protein with death domain
FLIP FLICE-inhibitory protein
FOXO Forkhead box protein O
GFP Green fluorescent protein
GSK-3β Glycogen synthase kinase 3β
HDAC Histone deacetylase
IKK IκB kinase
IL Interleukin
JNK c-Jun N-terminal kinase
KO Knockout
LTP Long term potentiation
MAPK Mitogen-activated protein kinase
MEF Mouse embryonic fibroblast
MEF2 Myocyte-specific enhancer factor 2
MHC Myosin heavy chain
MRF Myogenic regulatory factor
NER nucleotide excision repair
NF-κB Nuclear factor kappa enhancer binding protein
NGF Nerve growth factor
PAR-4 Prostate apoptosis response
PARP Poly (ADP-ribose) polymerase
PBS Phosphate-buffered saline
PDGF Platelet-derived growth factor
PDK1 3-phosphoinositide-dependent protein kinase 1
PFA Parafolmaldehyde
PI Propidium iodide
PI3K Phosphatidylinositol 3-kinase
PKC Protein kinase C
PS Pseudosubstrate
PTP Protein tyrosine phosphatase
RB Retinoblastoma protein
RIP Receptor interacting protein
ROI Reactive oxygen intermediate
SDS Sodium dodecyl sulphate
TNFα Tumor necrosis factor α
TNFR Tumor necrosis factor receptor
TRADD TNFR1-associated death domain protein
TRAIL TNF-related apoptosis inducing ligand
TRE TPA-response element
UV Ultraviolet
WT wild type

ABSTRACT

Stress signals are often sensed by membrane-bound proteins which translate signals into the chemical modification of molecules, most notably protein kinases. These kinases, in turn, transmit the decoded message through a cascade of sequential phosphorylation events to specific recipient transcription complexes which activate the appropriate gene expression profile.

One of the best known transcription factors targeted by stress signals is the AP-1 family member c-Jun which is essential for cellular adaptation to many environmental changes and oncogenic transformation. The specificity of the c-Jun-mediated biological response is provided by the assembly of diverse c-Jun-containing complexes, determined by distinct dimerization partners, post-translational modifications and availability of regulatory factors. Hence, upon a specific signal, only a small subset of all potential targets is controlled by Jun proteins in a particular cell type, depicting c-Jun as a multitasking, yet strictly regulated factor.

In this study, I have identified the nucleolar protein AATF as a novel regulator of c-Jun transcriptional activity. AATF is able to activate c-Jun in a JNK-independent manner, suggesting an involvement of AATF in the poorly understood phosphorylation-independent functions of c-Jun. Moreover, I provide evidence that AATF is confined to the nucleolus at normal growth conditions and that distinct stimuli result in its redistribution into the nucleoplasm where AATF is able to promote c-Jun activity. Importantly, I show that the AATF-mediated potentiation of c-Jun transcriptional activation leads to a prominent increase in apoptosis, likely mediated by the induction of the proapoptotic genes *FasL* and *Tnf-α*. My results are in line with current hypothesis suggesting that the strength of the stress signal determines the amplitude and intensity of the activation of the c-Jun response, in which a transient activation may be associated with a suppression of the UV-induced cell death by inducing cell cycle arrest, whereas prolonged c-Jun activation triggers apoptosis.

In parallel, I further characterized the Cdk5/ p35 signaling complex previously identified in our laboratory as an essential determinant of myoblast differentiation. I identified the atypical PKCξ as an upstream regulator of the Cdk5/ p35 complex during myoblast differentiation and show that the cleavage and activation of the Cdk5 regulator p35 is of physiological relevance for the differentiation process and dependent on PKCξ activity. I show that upon induction of differentiation PKCξ phosphorylates p35 on serine 33 and that this phosphorylation is necessary to allow the calpain-mediated cleavage of p35 and the consequent increase in Cdk5 activity. Finally, PKCξ phosphorylates also calpain during myoblast differentiation, suggesting that PKCξ functions both by turning p35 into a calpain cleavage-permissive form and by boosting calpain activity.
In summary, this PhD thesis expands the understanding of the regulatory mechanisms governing c-Jun transcriptional activity and c-Jun dependent apoptosis identifying AATF as a key determinant. Moreover, this work provides new perspective for the function of the Cdk5/ p35 complex during myoblast differentiation and identifies PKCξ as an upstream regulator of Cdk5 activity and of myoblast differentiation.

INTRODUCTION

Physical or chemical factors which change the existent equilibrium of a cell induce stress responses which aim at restoring cellular homeostasis. In a single yeast cell, stress responses induce a change in mating types which generate diversity by sporulation. This process is mediated by stress-activated kinases, including stress-activated protein kinase/ jun kinase (SAPK/ JNK), p38 mitogen activated protein kinase (p38 MAPK), extracellular response kinase (ERK)5, AMP-dependent kinase (AMPK), and Janus kinase (JAK). In mammals, homologous pathways regulate stress responses and are activated by many common stress inducers such as oxygen perturbation, serum starvation, cytokines and hyperosmolar stress, all of which induce cell cycle arrest and are followed upon persistent stress by either differentiation or cell death. Interestingly, many aspects of both cell differentiation and cell death are controlled by common molecular effectors. For example, both apoptosis and differentiation use caspases, cysteine aspartate proteases which cleave and degrade proteins to allow morphological changes necessary for the formation of a differentiated and highly specialized cell or to accomplish the regulated removal of a damaged cell.

The proper sensing and response to stress is necessary for a successful strategy in survival and readjustment of cellular homeostasis. The strategy of choice can differ between a unicellular and a multicellular organism, as an apoptotic response may be advantageous for a cell in a multicellular organism but deleterious for a unicellular organism. As a consequence, threshold levels for stress pathways activation and additional regulatory proteins have evolved in order to allow a more sophisticated and diversified response, and a less stringent cell death activation. In parallel, stress pathways have also differentiated with the progressive increase in cellular compartmentalization: new organelles and structures appear in eukaryotes or some organelles acquire new functions to allow the complex and highly specialized communal life of a multi-cellular organism. Among them, the nucleolus is a nuclear compartment dedicated to ribosome biogenesis and therefore directly connected to protein synthesis and cell growth and proliferation. Not surprisingly, several stress sensors, such as MDM2, SIRT7, VHL and ARF are found as permanent or signal-induced residents of this structure, allowing the cell to couple energy availability and metabolism to cell proliferation during adverse conditions. In the nucleolus we find also AATF, a transcription factor involved in rRNA processing, ribosomal biogenesis and stress response, which in this thesis I show to be a necessary regulator of the AP-1 transcription factor c-Jun and of c-Jun-mediated cell death response. Moreover, I demonstrate that the pro-apoptotic role of AATF is associated to its translocation from the nucleolus to the nucleoplasm, suggesting that AATF might play distinct roles in different sub-cellular compartments, such as nucleolar-associated functions and c-Jun transcriptional regulation in the nucleus.

Similarly, also the other stress and apoptosis regulator subject of this PhD study, the Cdk5/ p35 complex, changes localization upon distinct stimuli and thereby acquires distinct functions: in the cytoplasm Cdk5/ p35 is associated to the intermediate filament nestin, and regulates cytoskeletal rearrangements such as those promoting cellular migration, while upon a stress signal, it translocates into the nucleoplasm where it can promote cell death. Here I show that the Cdk5/ p35 complex translocates to the nucleus also upon induction of differentiation, identifying another common aspect of the differentiation and apoptotic pathways, and I show that this translocation is regulated by the atypical PKCξ. Indeed, I describe how, on one hand, PKCξ directly targets and activates the calpain protease activiys and how, on the other hand, PKCξ phoshorylates the calpain substrate p35, thereby enabling p35 cleavage and the consequent increase in Cdk5 activity. I demonstrate that both events,

the activation of calpains and of the Cdk5/p35 complex, are required for muscle differentiation and are dependent on PKCξ activity.

REVIEW OF THE LITERATURE

1. Apoptosis

Apoptosis is a form of regulated cell death which eliminates unwanted, damaged or superfluous cells from the organism. It plays an indispensable role during embryogenesis and in adult tissues. Deregulation of apoptosis can contribute to pathogenic states (Ellis, Yuan, & Horvitz, 1991; Raff, 1992; Raff et al., 1993; Steller, 1995). For example, excessive apoptosis is known to take place in various diseases, such as cerebral ischemia (reviewed by Broughton *et al*, 2009), AIDS (reviewed by Muthumani *et al*., 2003), and neurodegenerative diseases (Rohn *et al*., 2010). Conversely, defective apoptosis, causing an excessive cell number accumulation, is a key characteristic of cancer development (reviewed by Hanahan & Weinberg, 2000). Apoptosis is an energy-dependent process characterized by sequential steps of morphological changes (Wyllie, Kerr, & Currie, 1980). The dying cell undergoes nuclear and cytoplasmic condensation, blebbing of the plasma membrane, and fragmentation into membrane-enclosed apoptotic bodies. These bodies are subsequently identified and engulfed by phagocytic cells, such as neutrophils, monocytes, macrophages and dendritic cells. The endocytosis of apoptotic bodies avoids the induction of an inflammatory response, which instead characterizes the necrosis type of cell death, an uncontrolled process, in which the cell swells and releases the cell contents into surrounding tissues (reviewed by Golstein & Kroemer, 2007).

1.1. Apoptosis executers: the caspases

Caspases are cysteine proteases which cleave their substrate proteins at certain aspartate residues and are the main executers of the apoptotic pathway (Chowdhury, Tharakan, & Bhat, 2008). In most cell types, caspases are expressed as catalytically inactive zymogens, consisting of a prodomain, a large p20 subunit, and a small p17 subunit (Figure 1). The initial caspase activation process separates the large and small subunits, followed by removal of the N-terminal domain to form the catalytically active protease. Caspases are classically grouped into two classes: “initiator” caspases and “executioner” caspases. The main role of the initiator caspases is to cleave and activate executioner caspases, while the executioner caspases are responsible for the proteolytic processing of other proteins (Riedl & Shi, 2004). The initiator caspases, including caspase-2, -8, -9, and -10, have a longer N-terminal prodomain than the executioner caspases and contain one of two characteristic protein–protein interaction motifs, the caspase recruitment domain (CARD) (Caspase-2 and -9) or the death effector domain (DED) (caspase-8 and -9) (Figure 1). These interaction motifs allow the recruitment of procaspases to specific death signaling complexes through the interaction with adaptor molecules which contain similar interactive motifs. Indeed the mechanism of activation of initiator caspases relies on specific adaptor proteins which promote the assembly of a caspase activating complex in response to death stimuli, followed by recruitment of procaspases. The result is an increase in local concentration of the initiator procaspases which allow zymogen dimerization and subsequent autocatalysis (Pop, Fitzgerald, Green, & Salvesen, 2007).

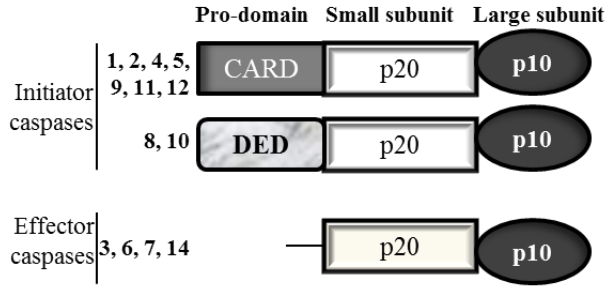

Figure 1. General structure and classification of caspases. Initiator caspases are characterized by a longer prodomain containing either a CARD interacting domain (caspase 1, 2, 4, 5, 9, 11, 12) or a DED domain (caspase 8, 10). Effector caspases are characterized by a short prodomain (caspase 3, 6, 7, 14)

In mammals, four specific caspase activating complexes have been described: 1) the apoptosome, which enables the activation of caspase-9 via interaction with the adaptor APAF-1 (apoptotic protease-activating factor-1) in the presence of cytochrome *c*; 2) the death-inducing signaling complex (DISC), which enables the activation of caspase-8 via interaction with the adaptor FADD; 3) the inflammasome, which regulates the activation of caspase-1 and caspase-5 via interaction with the adaptor, ASC (apoptosis-associated speck-like protein containing a CARD) or the family of NLRs (nucleotide-binding and oligomerization domain (NOD)-like receptors); 4) the PIDDosome, which regulates the activation of caspase-2 via interaction with the adaptors RAIDD (RIP (receptor-interacting protein)-associated ICH-1/ CED-3 homologous protein with a death domain; also called CRADD) and PIDD (p53-induced protein with a death domain).

The different caspase-activating complex are formed and activated in response to distinct pro-apoptotic and pro-inflammatory signals, which suggest that they perform specific biological functions. For example, the apoptosome assembles upon loss of mitochondrial integrity and consequent release of cytochrome *c*, whereas the DISCs is formed upon binding of the Fas ligand (Fas-L) or of the tumor necrosis factor-α (TNFα) to their respective death receptors, as described in more detail below. The executioner caspases, caspase-3, -6 and -7, need to be cleaved by the initiator caspases in order to turn on their protease activity. When an initiator caspase cleaves the target caspases into fragments of 20 (p20) and 10 (p10) kDa, it allows the association and formation of a tetrameric enzyme complex derived from the processing of two procaspase zymogens (Riedl & Shi, 2004). The active tetrameric protease consists of two homodimers formed by a large and a small subunit containing two active sites at opposite ends of the molecule (Figure 2).

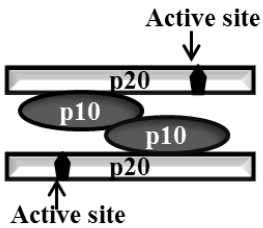

Figure 2. Schematic representation of a mature mammalian caspase. Initiator caspases cleave effector caspases into two fragments of 10 and 20 kDa, resulting in the exposure of the catalytically active cysteine, indicated by the black circle. The mature active effector caspase is a homodimer, with each monomer formed by a large and small subunit and containing the two active sites at the opposite ends of the tetramer (modified from Chowdhury et al., 2008).

In addition, active executioner caspases trigger DNA fragmentation through the cleavage-induced activation of the caspase-activated DNase CAD (caspase-activated DNAse) (Lüthi & Martin, 2007). Active caspases recognize and cleave proteins containing the peptide sequence X-Glu-X-Asp. Caspases-1, -4, -5 prefer the tetrapeptide WEHD. Caspases-2, -3, -7 prefer

DEXD, while caspase-6, -8, -9 prefer the sequence (L/ V)EXD. Active caspases associate to their substrates through interactions of the active site cleft with amino acids in the substrate cleavage site. Upon binding of the caspase to the substrate, the cleavage is accomplished by hydrolysing peptide bonds on the carboxyl side of the aspartate residue of the cleavage recognition motif X-Glu-X-Asp (Degterev et al., 2003). The sequential activation mechanism of caspases leads to the amplification of the original stress signal, which causes the cleavage of hundreds of structural and regulatory proteins and the consequent death of the cell (Lüthi & Martin, 2007).

While caspase activity is associated with the onset of apoptosis , some caspases have also non-apoptotic functions: Caspase-1 is critical for inflammatory responses (Martinon, Burns, & Tschopp, 2002); Caspase-8 and its adaptor FADD are essential for apoptosis induced by death-receptors but also for blood vessel development, macrophage differentiation and the proliferation of certain cell types (Thome & Tschopp, 2001); Caspase-3 activity is necessary for muscle differentiation (Fernando, Kelly, Balazsi, Slack, & Megeney, 2002), for differentiation of embryonic keratinocytes (Okuyama *et al*., 2004) and of some cancer cells such as K562 cells (Sztiller-Sikorska, Jakubowska, Wozniak, Stasiak, & Czyz, 2009).

1.2 Two convergent pathways lead to caspase activation: the intrinsic and extrinsic pathway

Vertebrates have two distinct apoptotic signaling pathways which ultimately converge in the activation of caspases: the intrinsic or mitochondrial pathway and the extrinsic or death receptor pathway. These pathways, and their most central regulatory proteins, are discussed below.

1.2.1 The intrinsic pathway

The intrinsic apoptotic pathway utilizes a mitochondrial signaling route and is triggered in response to many qualitatively distinct stimuli, including DNA damage, cytokine deprivation, and cytotoxic drugs. Activation of the intrinsic pathway leads to mitochondrial depolarization and release of cytochrome *c* from the mitochondrial intermembrane space into the cytoplasm through proteolipid pores formed by the proapoptotic members of the Bcl-2 protein family, such as Bax and Bak (Chipuk, Bouchier-Hayes, & Green, 2006). In intact mitochondria, cytochrome *c* functions as an electron transporter in the respiratory chain located in the mitochondrial inner membrane. Once released into the cytosol, cytochrome *c* associates with APAF-1 and procaspase-9 and forms a complex called the apoptosome. Here caspase-9 is activated and promotes in turn, cleavage and activation of the executioner caspase -3, -6 and -7 (reviewed by Riedl & Salvesen, 2007). The intrinsic pathway is regulated by interactions between proteins related to the Bcl-2 family members.

1.2.1.1. The Bcl-2 family

The members of the B-cell-lymphoma (Bcl) proteins, named after the human Bcl-2 oncogene (Tsujimoto et al., 1984), can be divided based on their pro- or anti-apoptotic role and based on the number of Bcl-2 homology (BH) domains they possess, into three interacting groups (reviewed in Martinou et al., 2011) (Figure3):

1) the pro-survival group, consisting of the Bcl-2 and its closest homologues, which share four BH domains;

2) the pro-apoptotic group, the Bax group, which are very similar in sequence and structure to the pro-survival group;

3) the apoptosis initiator group, the BH3-only proteins (Bad, Bid, Bim, Bik, Noxa and Puma) which possess only one BH domain, the so called BH3 domain (Adams & Cory, 2007).

Most Bcl-2 family members contain a C-terminal hydrophobic trans-membrane (TM) region, which mediates their targeting and anchoring to the mitochondrial outer membrane and/ or to the ER (Figure 3).

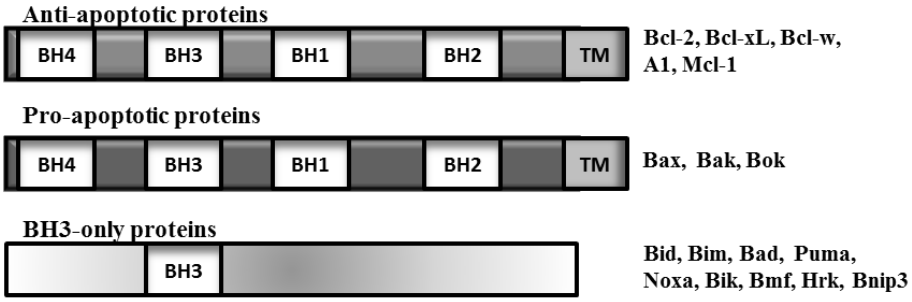

Figure 3. Structure of Bcl-2 family proteins. The Bcl-2 family is formed by three groups based on their Bcl-2 Homology domains (BH) and function. Pro- and antiapoptotic proteins have 4 BH domains, while BH3-only proteins, as their name indicates, have only the BH3 domain (adapted from Happo et al., 2012).

The BH3-only proteins initiate apoptosis by binding to the hydrophobic surface of the pro-survival family members and antagonizing their activities. The affinities of BH3-only proteins for pro-survival proteins differ markedly. BIM, tBID and PUMA bind with high affinity to all pro-survival proteins, while Bad binds with high affinity only to Bcl-2, Bcl-X_L and Bcl-W, and Noxa only to MCL1 and A1 (reviewed in Martinou *et al*., 2011). The activity of the BH3-only proteins is regulated both transcriptionally and post-transcriptionally through post-translational modifications. For example, the transcription of *noxa* and *puma* is induced by DNA damage and is regulated by the tumor suppressor p53, while *bim* transcription can be activated by the forkhead transcription factor FOXO3 upon cytokine withdrawal (Dijkers et al., 2000), by CHOP, a member of the C/ EBP transcription factor family, upon endoplasmic reticulum (ER) stress (Puthalakath et al., 2007), or by Myc (Egle et al., 2004). Bim is also regulated by sequestration in a microtubule-associated dynein motor complex in the cytoplasm of healthy cells and its transcription seems to be regulated by MAPK and PI3K pathways (Gross, McDonnell, & Korsmeyer, 1999). Bad is phosphorylated by Akt, and thereby kept sequestered in the cytoplasm by 14-3-3 (Zha, Harada, Yang, Jockel, & Korsmeyer, 1996). Upon stress, Bad is dephosphorylated and subsequently released from 14-3-3 proteins, enabling Bad to relocalize to mitochondria. In contrast, Bid is activated when cleaved by caspase-8 or caspase-10. The truncated Bid, tBid, can be myristoylated which is required for targeting tBid to the mitochondria (Puthalakath & Strasser, 2002). Importantly, the pro-apoptotic Bcl2 members Bax and Bak are necessary for mitochondrial apoptosis as double knock-out mice models show an impaired mitochondrial outer membrane permeabilization and resistance to a wide range of apoptotic stimuli (Wei et al., 2001). While Bax is predominantly a cytosolic monomer in healthy cells, during apoptosis it undergoes conformational changes, translocates to the outer mitochondrial membrane and oligomerizes. On the contrary, Bak is already localized at the mitochondrial membrane under normal growth conditions, but upon apoptotic stimuli it changes conformation and forms larger aggregates which permeabilize mitochondria and allow the passage of cytochrome *c*. Therefore, Bax and Bak together permeabilize the outer mitochondrial

membrane, allowing efflux of apoptogenic proteins and initiation of the caspase cascade. The precise mechanism by which Bak and Bax mediate mitochondrial apoptosis is still unclear, and will not be further discussed in this thesis (Green, 2006; Willis & Adams, 2005)

Caspase activation may further be regulated via binding to specific inhibitors, the IAPs (inhibitors of apoptosis). IAPs are a family of proteins, originally identified in insect cells infected by the baculovirus (Roy *et al.*, 1995, Cheng *et al.*, 1996 and Salvesen and Duckett, 2002). In human, six IAP members have been identified: NAIP (neuronal apoptosis inhibitory protein), c-IAP1, c-IAP2, XIAP (X-linked mammalian inhibitor of apoptosis protein), survivin and BRUCE (Deveraux and Reed, 1999). All IAP members contain one to three N-terminal baculovirus IAP repeat (BIR), a novel domain of 70–80 amino acids forming a zinc finger like structure which can chelate zinc ions. The BIR domains can bind to the surface of caspases and thereby inhibit caspases activities. The executioner caspases, caspase-3, -7 and -9 can all be inhibited by IAPs (Salvesen & Duckett, 2002). However, not all BIR-containing proteins are inhibitors of caspases. For example, survivin, which has only one BIR domain, seems to function as a regulator of mitosis rather than apoptosis. In turn, the activity of human IAPs can be inhibited by Smac/ Diablo proteins released by the mitochondria (Green & Kroemer, 2004; Jost et al., 2009)

1.2.2. The extrinsic pathway

The extrinsic apoptotic pathway is initiated by the binding of ligands to specific death receptors (DRs) on the plasma membrane. The activation of death receptors promotes the formation of cytoplasmic caspase-activating protein complexes, which will ultimately induce apoptosis (Aggarwal, 2003) (Figure 4). The extrinsic pathway is responsible for the elimination of unwanted cells during development, immuno-surveillance for tumor cells removal and the regulation of the immune system when stimulated upon death receptor ligation. To date, six DRs have been identified: TNFR1 (p55), CD95 (Fas/ Apo), DR3 (TRAMP), DR4 (TRAIL-R1), DR5 (TRAIL-R2) and DR6. DRs are activated by binding to their ligands which belong to the TNF superfamily of cytokines. Most death receptor ligands are expressed as type II transmembrane proteins predominantly on the cell surface of immune cells, while their respective receptors are expressed in a wide range of tissues as type I transmembrane proteins as preassembled homotrimers (reviewed by Nagata 1997). Death receptors can be divided into two types according to the adaptor protein to which they associate upon ligand binding. The adaptor proteins contain binding domains to the death receptors and docking sites for other downstream signaling molecules. Fas, TRAIL-R1 and TRAIL-R2 recruit the adaptor protein FADD and primarily mediate proapoptotic signals, whereas TNFR1 and TRAMP bind the adaptor protein TRADD and mediate mainly proinflammatory and immune-stimulatory activity (Hsu, Xiong, & Goeddel, 1995).

As an example of the first DR group, Fas is activated by binding to its ligand, FasL, which initiates the rapid clustering of the receptors (Figure 4). Fas recruits FADD and caspase-8 to form the death-inducing signaling complex (DISC), where caspase-8 and caspase-10 are activated by induced proximity (Medema et al., 1997; Muzio, Stockwell, Stennicke, Salvesen, & Dixit, 1998). Recruitment of c-FLIP to the Fas-DISC regulates caspase-8 activation and promotes anti-apoptotic Fas signaling (Thome & Tschopp, 2001). TNFR1 signaling represents the second DR group and is mediated by the formation of two different complexes which can either activate the NF-κB pathway from the cell surface (Complex I) or initiate apoptosis from the internalized receptors (Complex II) (Micheau & Tschopp, 2003) (Figure 4). Complex I forms upon binding of the ligand TNFα to the receptor TNFR1 which induces the recruitment of the adaptor protein TRADD and consequent binding of TRAF2, cIAP1, cIAP2

and RIP1. Here, RIP1 ubiquitination allows for activation of IKK complex which regulates the phosphorylation-mediated degradation of IkBα, the NF-κB inhibitor protein. The stabilized NF-κB translocates to the nucleus and induces the expression of antiapoptotic genes such as *cIAP-1*, *c-IAP-2*, *cFLIP*, *TRAF1* and *TRAF2* (Wilson, Dixit, & Ashkenazi, 2009). Alternatively, TNFR1 can be endocytosed upon TNFα binding. In this case, RIP1 is deubiquitinated and another complex, the pro-apoptotic Complex II (also called DISC), can form. This complex includes TRADD, FADD and procaspase-8. The complex II pathway resembles the pro-apoptotic cascades induced by Fas-L and is regulated by c-FLIP, c-IAPs and the deubiquitinating enzyme CYLD (Micheau & Tschopp, 2003; L. Wang, Du, & Wang, 2008; Wilson et al., 2009). Hence, it seems that the seemingly contradictory functions of the death receptors, such as survival and apoptosis, are mediated by the different receptor complex which can form either at the plasma membrane or when endocytosed into the intracellular compartments (reviewed in Schuetze *et al*. 2008).

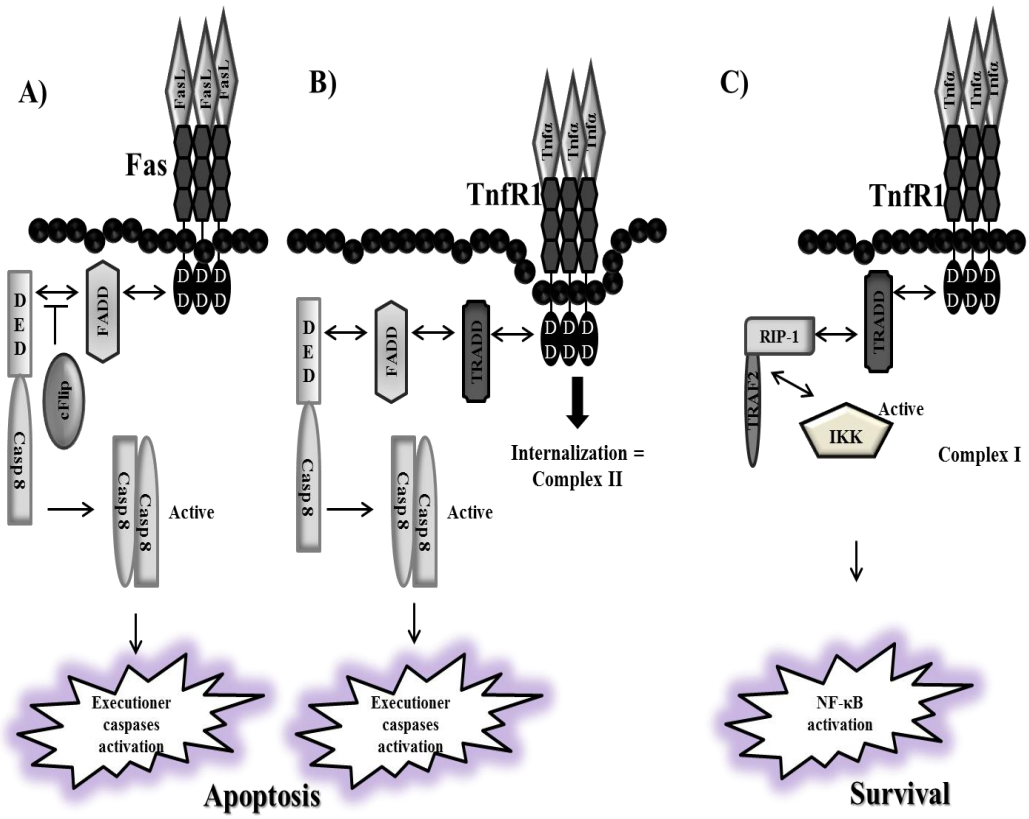

Figure 4. Death Receptor Complexes. (A) Formation of the death inducing signaling complex (DISC) via stimulation of Fas by its respective ligand FasL. Fas ligation triggers direct recruitment of the adaptor FADD which binds procaspase-8 (or procaspase-10) via its DED domain. Here initiator caspases are activated by induced proximity. c-Flip blocks this process through direct binding and inhibition of procaspase-8. **(B)** Tnfα ligation triggers receptor endocytosis, internalization and assembly of a caspase activation complex. Upon endocytosis the adaptor TRADD associates to TnfR1

and, than binds FADD through interaction between their DD domains. FADD in turn recruits procaspase-8 and/ or procaspase-10 and initiate apoptosis. **(C)** Binding of Tnfα to TnfR1 promotes the assembly of Complex I at the plasma membrane which consists of the adaptor protein TRADD, the kinase RIP-1 and the signal transducer TRAF2. IKK is recruited to complex I by RIP-1 and TRAF2 and is here activated. IKK activation leads to I-κB degradation and consequent NF- κB stabilization and activation.

1.2.2.1. Death ligands

Classically the activation of the extrinsic pathway relies on the expression level, availability and binding of the matching ligand to the correspondent receptor. The death ligands belong to the TNF ligand family of type II transmembrane proteins characterized by a conserved C-terminal extracellular TNF homology domain (THD) required for ligand trimerization. The ligands are synthetized as membrane-associated proteins, but they can be cleaved by proteolytic enzymes such as metalloproteases (Ethell, Kinloch, & Green, 2002; Mitsiades, Yu, Poulaki, Tsokos, & Stamenkovic, 2001). Some TNF ligands require solubilisation in order to function, whereas cleavage inhibits the physiological functions of others (Suda et al., 1997). Proteolytic shedding of TNFα (R. A. Black et al., 1997) and Fas-L produces soluble forms that might have a reduced ligand activity (Tanka et al., 1995; O´Reilly at al., 2009). Interestingly, death ligands can also activate signaling pathways that promote proliferation and survival. TNFα, for example, functions as a proinflammatory cytokine that induces proliferation and differentiation, but also mediates caspase activation and apoptosis in certain cell types (Aggarwal, 2003). The expression of *Fas-L* is tightly regulated at the level of transcription and can be induced by several transcription factors such as NFAT, NF-κB, c-Myc, IL-2, Sp-1, AP-1 and Egr (Kavurma & Khachigian, 2003). Upon stress *Fas-L* is induced by JNK members (Faris, Latinis, Kempiak, Koretzky, & Nel, 1998). Binding of Fas-L, TRAIL and TNFα to the correspondent receptor, also activates the JNK pathway (Y. Lin et al., 2000; Z. G. Liu, Hsu, Goeddel, & Karin, 1996; Wajant, Pfizenmaier, & Scheurich, 2003). Activation of the JNK pathway has been found to be necessary to Fas-mediated apoptosis but not for TNF-R1-induced cell death (reviewed in Liu and Lin, 2007). The best known targets of JNK are the AP-1 members ATF-2 and c-Jun that participate in the induction of *TNFα* and *Fas-L* following cellular stress or T cell activation.

1.3. The AP-1 transcription factors family

AP-1 (Activator Protein 1) was identified as a transcription factor that regulates both basal gene expression (W. Lee, Haslinger, Karin, & Tjian, 1987), and TPA (phorbol 12O-tetradecanoate-13-acetate)-inducible gene expression (Angel et al., 1987). However, AP-1 activity was soon thereafter shown to be potently induced also by several other types of stimuli, including serum stimulation (Lamph, Wamsley, Sassone-Corsi, & Verma, 1988), growth factors (Ryder & Nathans, 1988) (Quantin & Breathnach, 1988), oncoproteins (Angel & Karin, 1991; Mechta, Lallemand, Pfarr, & Yaniv, 1997), TNFα (Brenner, O'Hara, Angel, Chojkier, & Karin, 1989), and IL-1 (Muegge et al., 1989). The findings that growth factors and oncogenes induce AP-1 activity immediately suggested the importance of AP-1 in growth control and transformation (Jochum, Passegué, & Wagner, 2001). Also, the capacity of AP-1 to induce proinflammatory cytokines and the identification of the AP-1 target genes collagenase (Angel et al., 1987) and IL-2 (Muegge et al., 1989), suggested the involvement of AP-1 in inflammation and innate response. In addition to physiological stimuli, AP-1 activity is induced by a variety of environmental stresses, most notably short wavelength UV radiation (Devary, Gottlieb, Lau, & Karin, 1991; Herrlich, Ponta, & Rahmsdorf, 1992).

The mammalian AP-1 proteins form homodimers or heterodimers composed of basic region-leucine zipper (bZIP) proteins that belong to the Jun (c-Jun, JunB, and JunD), Fos (c-Fos,

FosB, Fra-1 and Fra-2) and the closely related activating factor (ATF2, ATF3 and B-ATF) subfamilies (Chinenov & Kerppola, 2001; van Dam & Castellazzi, 2001; Vinson et al., 2002). The less studied group of AP-1 proteins is the Maf subfamily that recent works have shown to play key roles in expression regulation, cellular differentiation and oncogenesis (reviewed in Kannan et al., 2012). Jun proteins can form stable dimers that bind the AP-1 recognition elements (5'TGAG/ CTCA-3'), known as TRE (TPA- response elements) (Angel et al., 1987). Fos proteins do not form stable homodimers but bind DNA through heterodimerization with the Jun proteins (Halazonetis, Georgopoulos, Greenberg, & Leder, 1988; Kouzarides & Ziff, 1988). ATF proteins can form both homodimers and heterodimers with the Jun proteins and preferentially bind to cAMP responsive elements (CRE, 5'- TGACGTCA-3') when dimerized with the Jun proteins. Thus the differential expression and dimerization partner of AP-1 proteins in response to extracellular stimuli is one major mechanism of regulation (Schütte et al., 1989). Indeed, the different components forming the AP-1 complex determines the binding specificity and consequently the spectrum of AP-1 targeted genes. Additionally, post-translational modifications, in particular phosphorylation, further modulate AP-1 activities and provide another link for extracellular stimuli to regulate AP-1 transcriptional activity (M Karin, 1995). Practically, all branches of the MAPK cascades have been found to be involved in the regulation of AP-1. Serum and growth factors induce AP-1 activities by activating the ERK branch of MAPKs, whereas pro-inflammatory cytokines and genotoxic stress induce AP-1 mostly by the JNK and p38 MAPKcascades (Chang & Karin, 2001; M Karin, 1995; Kyriakis & Avruch, 2001). Once activated, MAPKs translocate into the nucleus where they can directly phosphorylate and activate the AP-1 transcription factors. The primary effect of the MAPK-mediated phosphorylation is the potentiation of AP-1 transactivation without having a considerable effect on their DNA-binding ability (Karin, 1995). In this thesis, I focus on the c-Jun AP-1 family member and specifically on the role of c-Jun in cell death regulation.

1.3.1. c-Jun

c-Jun is, together with the Fos proteins, the major member of the AP-1 family. *c-jun* was identified in 1987 by its homology to v-*jun*, the oncogene found in the avian sarcoma virus ASV17 (Maki, Bos, Davis, Starbuck, & Vogt, 1987). A few years later, two other c-*jun*-related genes, *junB* and *junD*, were identified based on the sequence similarity to c-Jun (Hirai, Ryseck, Mechta, Bravo, & Yaniv, 1989; Ryder, Lanahan, Perez-Albuerne, & Nathans, 1989). Jun proteins can function as homodimers or as heterodimers bound to Fos-related proteins, CREB or ATF-2 (Hai & Curran, 1991; van Dam et al., 1993). The dimerization is mediated by a C-terminal coiled-coil leucine zipper that is necessary for binding to the palindromic sequence of the AP-1 consensus site, also known as the TPA-responsive element (TRE) (reviewed in Vogt and Bos, 1990, Angel and Karin, 1991). Therefore, different Jun homodimers and heterodimers form a large number of dimer combinations with distinct transcriptional properties and different biological outcomes. For example, the importance of the dimerization partner of c-Jun for its transformation capacity was shown by the demonstration that c-Jun-ATF2 heterodimers mediated the induction of autocrine growth while c-Jun-Fos heterodimers promoted anchorage-independent growth (Van Dam et al., 1998).

c-Jun is composed of a N-terminal region containing a JNK binding site and the transactivation domain (aa 1-191), a positively charged domain (aa 257-276), the leucine zipper (aa280-308) and a C-terminal region (Figure 5). Changes in the activation of c-Jun transcriptional activity depend on the chemical modification at three different sites:
1) the phosphorylation of the N-terminal region serine 63 and 73 (Ser63&73) and threonine 91 and 93 (Thr91&93) (Morton, Davis, McLaren, & Cohen, 2003; Smeal, Binetruy, Mercola,

Birrer, & Karin, 1991); 2) the dephosphorylation of Thr239 (Morton et al., 2003); 3) the acetylation of lysine residues in the basic, near C-terminal region aa 257-276 (Wang *et al*., 2006).
These modifications are under a direct or indirect control of the JNKs, the ERKs and the p38 MAPK components of the MAPK family.

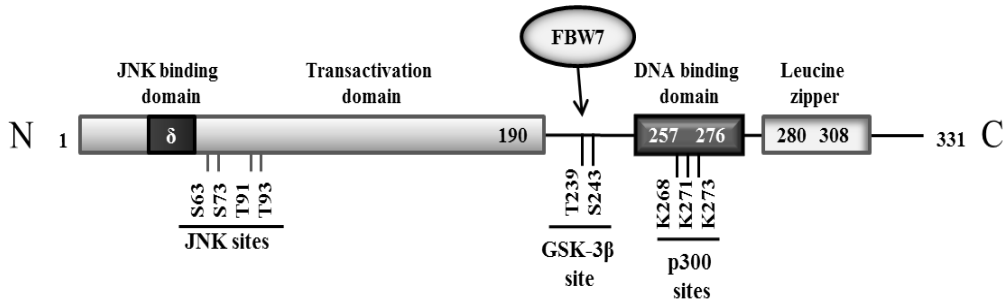

Figure 5. Schematic structure of human c-Jun. The N-terminal domain contains the JNK binding domain (δ) and the phospho-sites targeted by JNK (Ser63, 73 and Thr91, 93). Amino acids 1- 190 form the transcriptional transactivation domain. GSK-3β mediated phosphorylation of Thr239, together with Ser243 attracts the FBW7 ubiquitin ligase and targets phosphorylated c-Jun to ubiquitination and degradation. The DNA binding domain is a basic positively charged domain (aa 257-276) followed by the leucine zipper domain which mediates c-Jun dimerization. P300-mediated acetylation of the DNA binding domain results in JNK-independent activation of c-Jun.

Phosphorylation of c-Jun on Thr239 and Ser243 function as a docking site for the F-box&WD domain repeated 7 (FBW7) ubiquitin ligase which mediates the subsequent degradation of c-Jun (W. Wei, Jin, Schlisio, Harper, & Kaelin, 2005). Ser243 phosphorylation primes c-Jun for glycogen synthase kinase-3 (GSK-3)-mediated phosphorylation of Thr239 site (Morton et al., 2003). The phosphorylation of Thr239 is blocked by the substitution of Ser243 with phenylalanine in the transforming, viral form of the protein and causes the stabilization of viral Jun protein (v-Jun) (Boyle et al., 1991; W. Wei et al., 2005). The phosphorylation of both sites, Thr239 and Ser243, is required for binding of FBW7, so loss of both negative charges in v-Jun, or just one at Thr239 upon GSK3 inhibition, seems to be sufficient to stabilize c-Jun. Interestingly, abrogation of JNK-targeted phospho-sites, i.e. Ser63/ 73 and Thr91/ 93, also block FBW7-mediated degradation of c-Jun independently of the Thr239 and Ser243 pair, suggesting that the N-terminal phospho-sites constitute an alternative degradation pathway for the preferential removal of the phosphorylated and activated c-Jun (Nateri, Riera-Sans, Da Costa, & Behrens, 2004). Interestingly, the v-Jun form also carries a large deletion of aa32-58 in the JNK docking domain which precludes JNK binding and N-terminal phosphorylation (May et al., 1998). This deletion could explain why v-Jun only depends on the mutation of the Thr239/ Ser243 pair in order to escape FBW7-mediated degradation. Finally, post-translational modification in the C-terminal basic region can strongly affect c-Jun transcriptional efficiency. Upon certain stimuli, such as growth factor treatment, c-Jun activation depends on p300-mediated acetylation of lysine residues in the basic DNA binding domain (Vries et al., 2001; Y.-N. Wang, Chen, & Chang, 2006). For example, Wang and coworkers showed that substitution of the three lysines Lys268, Lys271 and Lys273 with arginine residues completely inhibits the epidermal growth factor-mediated activation of c-Jun, indicating that upon distinct stimuli c-Jun activity depends from the acetylation of its

basic C-terminal domain. By far the most well-known and potent activator of c-Jun transcriptional activity is the phosphorylation of the N-terminal domain operated by the JNK kinases, discussed here below.

1.3.1.1. The JNK pathway

Three different genes (*JNK1, 2, 3*) are alternatively spliced to form at least ten JNK isoforms (reviewed in Barr & Bogoyevitch, 2001; Dhanasekaran & Reddy, 2008). JNK1 and JNK2 are ubiquitously expressed, while the expression of JNK3 is mainly restricted to the brain and to a lesser extent to the heart and testis. The activation of JNK is mediated by the MAP cascade of sequential phosphorylations in which MAP3K phosphorylates and activates a MAP2K and which in turn phosphorylates MAPK (Figure 6). Two MAP2Ks (the JNKK1/ MKK4/ SEK1 and JNKK2/ MKK7) phosphorylate the MAPK subfamily JNK on the T-loop at Thr183 and Tyr185 and these phosphorylation events are required for JNK full activation (Dérijard et al., 1994; A. Lin et al., 1995; Sánchez et al., n.d.). JNK activation is also tightly regulated by many scaffolding proteins such as JIP, b-arrestin and JSAP, (M. Ito et al., 1999; Whitmarsh, Cavanagh, Tournier, Yasuda, & Davis, 1998) and MAP phosphatases (M Karin, 1995). Lastly, recent studies have shown that JNK activity can be negatively or positively regulated by NF-κB for inhibition or promotion of cell death induced by TNFα and UV, respectively (J. Liu et al., 2006; G. Tang et al., 2001). In addition, JNK phosphorylates and regulates the activities of other transcription factors such as ATF-2, Elk-1, p53 and c-Myc (Buschmann et al., 2001; Fuchs et al., 1997; Noguchi et al., 1999) and is involved in the regulation of non-transcription factors such as the members of the Bcl-2 family, Bim and Bad (Chang & Karin, 2001; Davis, 2000). Upon binding to c-Jun, JNK phosphorylates two serine/ threonine clusters, Ser 63/ 73 and Thr 91/ 93, and thereby potentiates c-Jun transcriptional capacity without having a significant effect on its DNA binding capacity (M Karin, 1995; Musti, Treier, & Bohmann, 1997) (Figure 6). JNK1 is the major isoform responsible for c-Jun terminal phosphorylation that is also the major isoform activated by UV-irradiation (Devary et al., 1991; J. Liu, Minemoto, & Lin, 2004; Sabapathy & Wagner, 2004). On the contrary, JNK2 seems to function mainly to target c-Jun for ubiquitination and degradation under normal growth conditions (Fuchs, Dolan, Davis, & Ronai, 1996). How JNK1 and JNK2 are differentially regulated is not yet understood. JNK phosphorylation controls c-Jun activity also by modulating the interaction between c-Jun and transcriptional co-repressors such as histone deacetylase 3 (HDAC3) or by regulating the nuclear localization of AP-1 regulators, such as the RNA helicase RHII/ Gu (Weiss et al., 2003; Westermarck et al., 2002). In addition, JNK1 phosphorylation has been suggested to increase the half-life of c-Jun, enabling the robust and prolonged expression after exposure of cells to stress such as UV radiation (Musti et al., 1997).

1.3.2. c-Jun role in apoptosis

The consequence of AP-1 activation is cell type specific (Potapova et al., 2001; Scherer et al., 2000; Wisdom et al., 1999). The biological outcome of the activation of the AP-1 complexes is highly dependent on the composition, post-translational modifications and presence of interacting factors within the complex. The clearest demonstration of the involvement of AP-1 in promoting apoptosis came from studies on the nervous system. Inhibition of c-Jun activity with a dominant negative mutant lacking the c-Jun N-terminal activation domain or by neutralizing antibodies, reduced apoptosis in neuronal cells induced by nerve growth factor (NGF) deprivation or chronic depolarization (Ham et al., 1995; Le-Niculescu et al., 1999; Xia, Dickens, Raingeaud, Davis, & Greenberg, 1995). Conversely, overexpression of c-Jun induces apoptosis in several cell lines (Bossy-Wetzel, Bakiri, & Yaniv, 1997; Ham et al., 1995). Also, expression of the phosphorylation-deficient c-Jun S63/ 73A mutant blocked apoptosis induced by NGF withdrawal in cultured neuronal cells as efficiently as treatment

with several JNK inhibitors, indicating that phosphorylation of c-Jun is necessary to promote c-Jun-mediated apoptosis (Le-Niculescu et al., 1999; Watson et al., 1998). The dominant negative c-Jun mutant also reduced apoptosis in human monoblastic leukemia cells after exposure to various DNA damaging agents (Verheij et al., 1996). Accordingly, both c-Jun-deficient fibroblasts and JNK1/ JNK2-deficient fibroblasts are resistant to apoptosis induced by UVC radiation (Shaulian et al., 2000; Tournier et al., 2000). However, only persistent, but not transient JNK activation promotes apoptosis (Hibi, Lin, Smeal, Minden, & Karin, 1993) (Chen, Wang, Templeton, Davis, & Tan, 1996). Indeed, persistent JNK activation in cells exposed to moderate to high levels of UVC (12-40 J/ m2) can result in a c-Jun induction lasting 24 h or longer (Shaulian et al., 2000). Moreover, it seems that *c-Jun* induction is necessary for cell cycle re-entry of UV-irradiated fibroblasts; indeed, while wild type fibroblasts undergo a transient cell cycle arrest after UV exposure, *c-jun* deficient cells undergo prolonged growth arrest and fail to resume proliferation (Shaulian et al., 2000). Conversely, cells that constitutively express c-Jun fail to arrest and continue to cycle after UV exposure.

It is likely that the pro-apoptotic effects of AP-1 are due to transcriptional activation of AP-1 target genes which promote apoptosis (Figure 6). For example, several studies have shown that AP-1 induces expression of the pro-apoptotic genes Fas-ligand (*Fas-L*), *Tnfα* and *Bim* (Kasibhatla et al., 1998; Kolbus et al., 2000; Le-Niculescu et al., 1999). Moreover, c-Jun KO fibroblasts, which are resistant to apoptosis induced by UV radiation and alkylating agents, show an impaired expression of *Fas-L* (Kolbus et al., 2000). However, there is no clear explanation why some activators of AP-1 lead to *Fas-L* induction and others not. It has been suggested that AP-1 plays a homeostatic function, which allows adjustment of the gene expression profile to changes in the environmental conditions, and enable the cell to adapt to the new environment. Moreover, upon excessive environmental stress, other stress pathways could be induced which may interact with the AP-1 complexes and promote cell death.

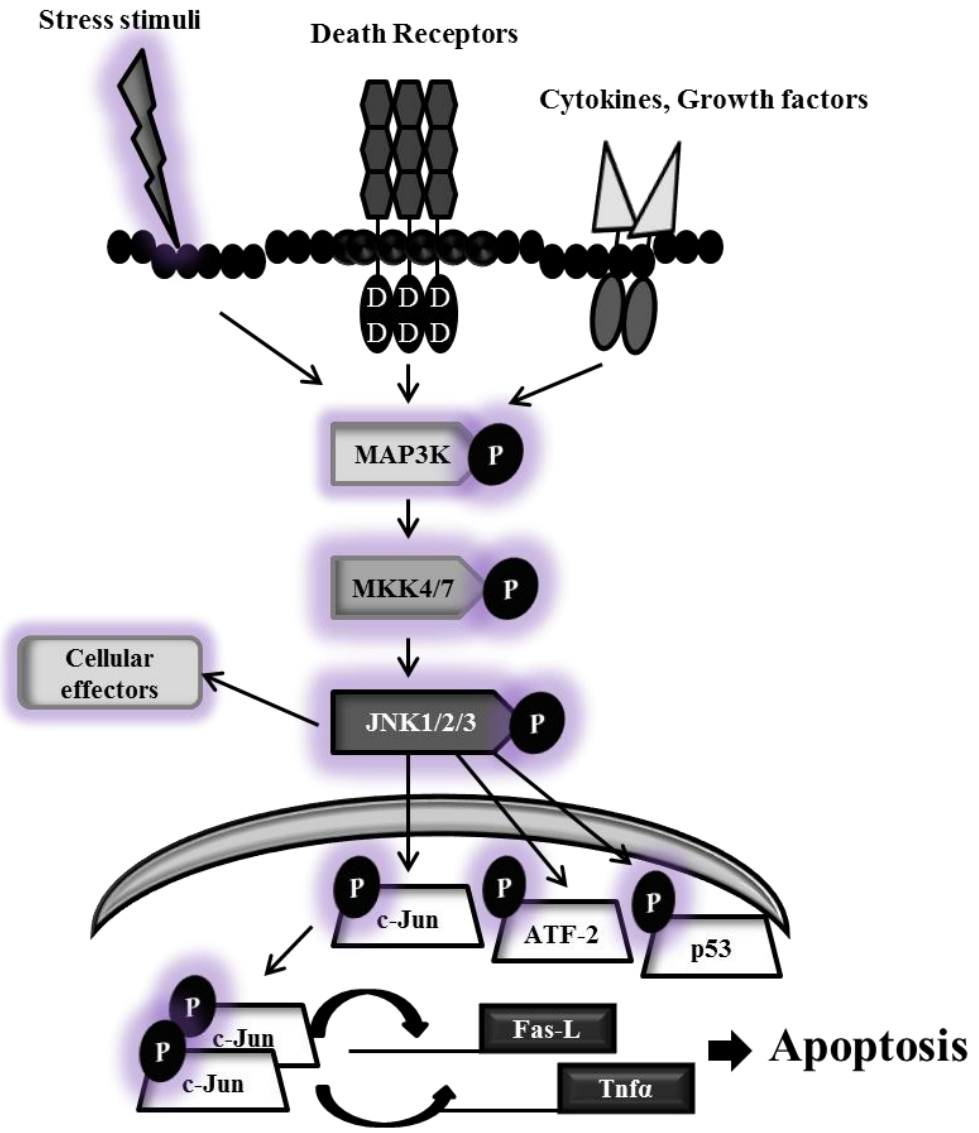

Figure 6. JNK signaling pathway in apoptosis. JNK is activated by a wide range of extracellular stimuli through the MAPK module consisting of a sequence of phosphorylation events. JNK is selectively phosphorylated by MKK4 and MKK7. Upon activation JNK targets Bcl-2 proteins, such as Bax, Bim and Bid, and translocates into the nucleus where it phosphorylates and contributes to stabilize p53 and activates the AP-1 factors c-Jun and ATF-2. Activated c-Jun dimers induce the proapoptotic genes *Fas-L* and *Tnfα* which are thought to be important mediators of c-Jun-mediated apoptosis. It is likely, however, that additional unidentified gene targets also contribute to apoptosis.

1.4. The UV response

UV exposure results in the simultaneous activation and inhibition of multiple signaling pathways which play critical roles in cell-type specific control of survival or death. The main source of UV radiation is sunlight. The shortest wavelength, UV-C (240-290 nm) is absorbed by atmospheric ozone, while 90% of the UV radiation which reaches the surface of the Earth is constituted by UV-A (315-280 nm) and approximately a 10% by UV-B (280-315 nm). However, the proportion of shorter wavelengths is increasing due to ozone depletion. Cells contain photosensitive molecules (chromophores) which, upon receiving photons from UV radiation, subsequently lift electrons to a higher and more reactive energetic state. The main cellular chromophores for UV radiation are DNA and reactive oxygen-generating molecules (Ravanat, Douki, & Cadet, 2001). Due to the aromatic ring structures of its bases, DNA absorbs short-wavelength very efficiently, subsequently forming pyrimidine dimers and (6-4)-photoproducts which cross-link adjacent DNA bases (Cadet, Sage, & Douki, 2005). These UV-induced distortions in the DNA helix block RNA polymerase elongation, thus inhibiting gene expression. The helix-distorting DNA adducts are repaired by nucleotide excision repair (NER) system whose effectors have been named according to seven complementation groups of Xeroderma pigmentosum (XP, XPA to XPG) (reviewed in Hoeijmakers, 2001). UV radiation causes also the accumulation of reactive oxygen intermediates (ROI), short lived intermediates including singlet oxygen, superoxide anion radical, hydroxyl radical. ROIs accumulation results in lipid peroxidation and also oxidize DNA sugar derivatives, modify bases, and cause single- and double-strand breaks (H. S. Black, 2004; Epe, 1996).

The primary DNA damage sensors are the phosphoinositide-3-kinase (PI-3-kinase)-related proteins ataxia telangiectasia-mutated (ATM) and ataxia telangiectasia-related (ATR), which have overlapping functions. ATM is essential for IR-induced and ATR for UV-induced phosphorylation of several downstream G1/ S checkpoint proteins, such as the Chk1 and Chk2 kinases (Kastan & Bartek, 2004; Shiloh, 2003). These, in turn, phosphorylate the Cdc25A phosphatase, leading to Cdc25A degradation and cell cycle arrest. ATM/ ATR kinases also phosphorylate the tumor suppressor p53, thereby stabilizing it (reviewed in Appella & Anderson, 2001; Kruse & Wei, 2009). UV-induced protein oxidation can initiate cell signaling by inactivating protein tyrosine phosphatases (PTPs) (Tonks, 2006). PTPs contain a highly reactive cysteine in their catalytic pocket. Oxidation of this cysteine inactivates PTPs and results in elevated phospho-tyrosine-dependent signal transduction. For example, upon PTP oxidation, firing of the EGF receptor is increased (Knebel, Rahmsdorf, Ullrich, & Herrlich, 1996; Sachsenmaier et al., 1994). MAPK phosphatases (MKPs), a specialized class of PTPs can also be similarly oxidated, resulting in a prolonged activation of MAPKs, especially JNK and p38 (Hamdi et al., 2005). As a third example, inactivation of PTEN, a PIP3 phosphatase, increases signaling by decreasing PIP3 turnover (S.-R. Lee et al., 2002). As a result, UV radiation provokes the clustering and internalization of several cell surface growth factor and cytokine receptors in a ligand-independent manner, resulting in the activation of downstream signaling cascades, a reason why the UV-response has been regarded as a "pseudo growth response" (Herrlich, Blattner, Knebel, Bender, & Rahmsdorf, 1997; Herrlich et al., 1992). A well-know MAPK cascade activated by UV exposure is the JNK and p38 kinase which, importantly, are only transiently induced and lead to AP-1 activation (Hildesheim & Fornace, 2004; Rosette & Karin, 1996).

The induction of cell cycle arrest upon DNA damage gives the cells time for DNA repair before replication resumes. The key regulatory protein involved in the induction of growth arrest by UV is p53 (Toledo & Wahl, 2006). Exposure to UV results in rapid p53

accumulation, caused by the stabilization of this otherwise short-lived protein. p53 accumulation causes the induction of several p53 target genes including *p21, Mdm2, Gadd45* and *Bax*. p21 is an inhibitor of cyclin-dependent kinases (Cdks) and is responsible for the induction of cell cycle arrest upon UV exposure. Importantly, DNA damage induces the rapid nucleolar translocation of HDM2 which targets p53 to degradation, and is essential for a proper DNA damage response through stabilization of p53 levels (Kurki et al., 2004; Rubbi & Milner, 2003).

2. Anti-Apoptosis-Transcription-Factor, AATF

Anti-Apoptosis-Transcription Factor (AATF / Che-1) is a RNA polymerase II binding protein involved in the transcriptional regulation of E2F target-genes and in cell proliferation. It does so by directly binding to the retinoblastoma protein (Rb) and inhibiting its ability to suppress expression of E2F genes by removing HDACI from E2F-target promoters (Tiziana Bruno et al., 2002; Fanciulli et al., 2000). However, AATF also has anti-proliferative activity as it can directly bind to the *p21* promoter and induce *p21* expression (Tiziana Bruno et al., 2006). Accordingly, in human colon carcinoma cell lines, overexpression of AATF induces cell cycle arrest by upregulating p21 expression in a p53-independent manner (Tiziana Bruno et al., 2006; Di Padova et al., 2003). Consistently AATF is downregulated in several tumors, including kidney, prostate and colon carcinoma (Di Padova et al., 2003) .On the *p21* gene promoter, AATF displaces HDAC1 from the Sp1 binding sites, suggesting that AATF might act as a general HDAC1 competitor and as an activator of transcription (Di Padova et al., 2003).

AATF is a highly conserved protein from yeast to mammals and its expression during embryogenesis is necessary for the preimplantation of the embryo (Thomas, Voss, Petrou, & Gruss, 2000). Indeed, the targeted disruption of AATF is lethal as it prevents the estabilishment of an embryonic gene expression profile and leads to the decompactation of the embryo. AATF-deficient mouse embryo is characterized by a 50% reduction in the total cell number and cells contain a reduced number of ribosomes. This paucity of ribosomes and the localization of AATF to the nucleoli, the site of ribosome synthesis, suggest that AATF might be involved in the biogenesis of ribosomes (Thomas et al., 2000).

Furthermore, AATF was identified as an interacting factor of DAP like kinase (Dlk), a member of the DAP (death-associated protein) kinase family of pro-apoptotic serine-threonine kinases and was named based on its capacity to antagonize Dlk-induced apoptosis (Page, Lödige, Kögel, & Scheidtmann, 1999).

2.1. Structure and physiological functions

AATF was originally identified in a two-hybrid screen as a binding protein of RNA polymerase II (Fanciulli et al., 2000). Soon after, Monaco and coworkers (2003) cloned the murine *AATF*, which encodes a protein of 526 amino acids but lacks the corresponding human exon coding for amino acids 146-177. In this work, the authors also showed that the mouse coding sequence of AATF consists of 1581 bp, subdivided into 13 exons spanning 35 kb on chromosome 17q11.2-q12, and that the AATF promoter region does not contain any TATA or CAAT boxes (Monaco *et al.*, 2003). Interestingly, AATF expression was shown to be regulated by a negative feedback mechanism, as AATF associated to its own promoter and, thereby, repressed its own transcription.

AATF is ubiquitously expressed, with the highest expression in the heart, brain, lung and testis (Monaco, Passananti, & Fanciulli, 2003). Three different splicing isoforms of AATF have been detected. Isoform 1 corresponds to the full length form of 1581 bp, whereas isoform 2 lacks exons III-IV (amino acids 85-199) and isoform 3 consists of a differently spliced C-terminal tail which lacks exons XI-XIII (amino acids 459-526) and encodes a

different exon XI translating amino acids 433-458 (LLRELIERKTSSLDPNDQVAMGRQWL →VRLFLSFLCYNKPGVCILEPLIVSSG) as summarized in Figure 7.

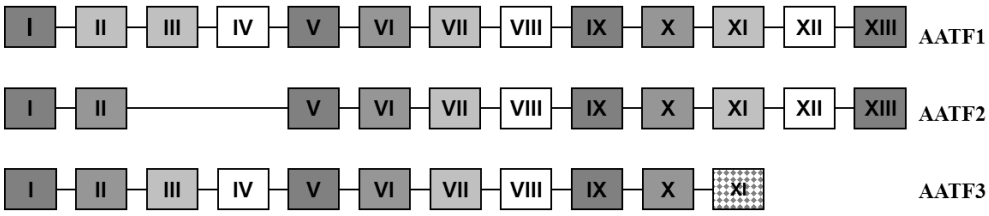

Figure 7. Murine AATF isoforms. Full length AATF (AATF1) is formed by 13 exons corresponding to a coding sequence of 1581 bp. Alternative splicing generates an AATF isoform missing exons III-IV (AATF2) or a differently C-terminal sliced variant (AATF3) lacking exons XI-XIII and encoding a different exon XI (AATF3).

Inspection of the amino acid sequence reveals the presence of a canonical leucine zipper motif, two highly acidic amino-terminal regions which are characteristic of several chromatin remodelling factors, such as VP16 and BRCA-1, various phosphorylation sites and three nuclear receptor binding LxxLL consensus sequences (Heery, Kalkhoven, Hoare, & Parker, 1997) (Figure 8). Two putative nuclear localization signals are present and indeed AATF is mainly localized in the nucleus and nucleoli (Andersen et al., 2005). Based on its capacity to directly associate with the core subunit 11 (RPB11) of human PolII and to transactivate a Gal4-linked reporter, AATF is thought to promote cellular transcription as an adaptor or co-factor (Fanciulli et al., 2000). Through its LxxLL motif, AATF can also bind directly to nuclear hormone receptors and thereby enhance the activity of various steroid receptors, such as androgen, estrogen, and glucocorticoid receptors in a hormone-dependent manner (Burgdorf, Leister, & Scheidtmann, 2004).

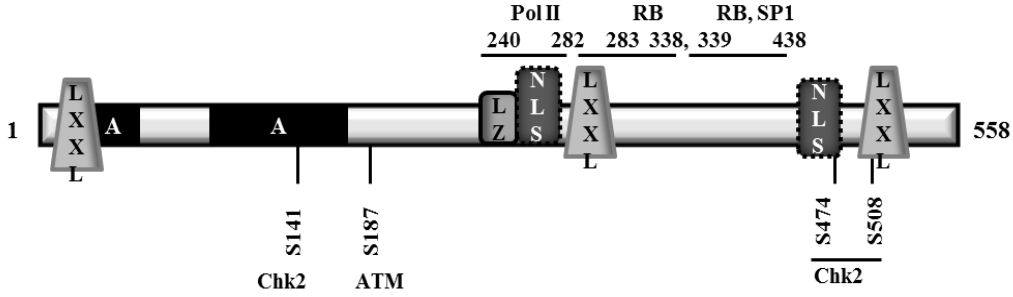

Figure 8. A schematic diagram of human Che-1/AATF protein structure. The N-terminal black boxes (amino acids 21-49 and 106-170) indicate highly acidic regions (A) which are separated by a Ser/ Thr-rich domain. The LZ box indicates the leucine zipper domain located between amino acids 239-260 (LZ). AATF contains 3 LXXL motifs, interaction motifs of coactivators for nuclear hormone receptors. The NLS boxes show putative nuclear localization signals. Phosphorylation of S141, S187, S474 and S508 by Chk2 and ATM/ ATR kinases promotes binding to *p53* and *p21* promoter and induction of their respective genes. AATF binding regions to Pol II (amino acids 240-282), RB (amino acids 283-338) and RB or SP1 (amino acids 339-438) are indicated.

AATF also plays an important role in the DNA damage response. Upon genotoxic stress, ATM/ ATR and Chk2 phosphorylate human AATF, thereby protecting it from degradation (Tiziana Bruno et al., 2006). ATM/ ATR targets Ser187 of AATF whereas Chk2 phosphorylates Ser141, Ser474 and Ser508 (Figure 7). At the same time, phosphorylation of these sites promote AATF binding to the *p21* and *p53* promoters and consequently promote the transcription of these genes (Tiziana Bruno et al., 2006). Although phosphorylation of these sites is required for recruitment of AATF to the *p53* promoter, they are dispensable for AATF binding to the E2F-target gene promoters. It has therefore been suggested that a pathway involving AATF, p53, and p21 might be a key component of the DNA damage checkpoints. However, a recent study showed that upon a genotoxic insult AATF phosphorylation by ATM/ ATR induces its translocation from the cytoplasm into the nucleus where AATF was shown to bind and inhibit the induction of p53 targeted genes *Bax*, *Bak* and *PUMA* (Höpker, Hagmann, Khurshid, Chen, Hasskamp, et al., 2012).

2.2. AATF role in apoptosis

AATF was named based on its capacity to inhibit apoptosis. Indeed, it seems that AATF has the capacity to bind and regulate the activity of several apoptotic factors. The anti-apoptotic effect of AATF was initially described through its antagonism of the DAP-like kinase (DLK) family of pro-apoptotic serine-threonine kinases (Page et al., 1999). DLK, also called ZIP kinase, is found tightly associated with nuclear PML bodies or with cytoplasmic actin filaments, where it induces apoptosis by enhancing the activity of Par-4, a leucine zipper protein which promotes neuronal death and aberrant production of β-amyloid peptide (Aβ) (Guo & Xie, 2004). However, AATF may also antagonize Par-4 function, as the binding of AATF to Par-4 inhibits secretion of Aβ in human neuroblastoma cells. Accordingly, AATF is found primarily mislocalized in the cytoplasm in neuronal tissues from Alzheimer's patients (Guo & Xie, 2004). AATF also counteracts the activity of neurotrophin receptor-interacting MAGE homolog (NRAGE), a cytoplasmic protein involved in the regulation of apoptosis during neuronal development (Di Certo et al., 2007). In this case, NRAGE retains AATF in the cytoplasm and targets it for proteasome-dependent degradation. At the same time, AATF inhibits NRAGE-induced cell death, acting as a functional antagonist of NRAGE. AATF induces also the expression of Akt by cooperating with Stat3 during endoplasmic reticulum stress (ER stress) and thereby promotes survival (Ishigaki et al., 2010). Another example in which AATF acts as an anti-apoptotic factor, is via induction of XIAP expression (Bruno et al., 2008). As demonstrated by Bruno and coworkers, in response to genotoxic stress AATF collaborates with NF-κB to induce XIAP expression and this activity is required for the anti-apoptotic role of AATF in human colon carcinoma cell lines (Bruno et al., 2008). Lastly Höpker and coworkers have shown that AATF prevents apoptosis by inhibiting induction of p53-targeted genes *PUMA, Bax* and *Bak* by directly binding to their respective promoters (Höpker, Hagmann, Khurshid, Chen, Schermer, et al., 2012).

3. Myoblast differentiation

Skeletal muscle differentiation is a complex, multistep process characterized by the irreversible withdrawal of proliferating myoblasts from the cell cycle, the expression of muscle-specific genes, migration and fusion of plasma membranes to enable the formation of multinucleated myotubes (Figure 8). In adult muscle tissue, myoblast proliferation, fusion and differentiation are reactivated upon injury and mimic the basic steps characterizing embryonal myogenesis.

3.1. Myogenesis – an overview

Myogenic precursors are specified during development by signals emanating from neighbouring cells of the notochord, neural tube, and dorsal ectoderm via the WNT and Sonic Hedgedog pathways as positive regulators, and via the BMP pathway and the Notch pathway as negative regulators. This specification depends critically on the function of myogenic transcription factors, such as Pax3 and Pax7 (Borycki, Li, Jin, Emerson, & Epstein, 1999; Cossu, Tajbakhsh, & Buckingham, 1996). Once committed, cells derived from the somites, which are cell clusters generated by segmentation of the paraxial mesoderm, migrate to several sites of embryonic myogenesis, where they begin to express the myogenic basic helix-loop-helix transcription factors Myf5 and MyoD (Birchmeier & Brohmann, 2000) and to differentiate into muscle fibers. Development of mouse limb muscles is characterised by two sequential waves of proliferation and migration of myoblasts, as well as fusion and differentiation in muscle fibers. In the first stage, occurring between embryonic day (E) 11 and E14, the first muscle fibers, commonly referred to as primary fibers are formed (Buckingham et al., 2003). Several days later, during fetal development, secondary muscle fibers are added and allow muscle growth to proceed. The secondary fibers derive from the proliferation of either a previously quiescent population of myoblasts (Cusella-De Angelis et al., 1994) or a population of Pax3-Pax7-positive progenitor cells (Relaix, 2006). Most likely, the somite-derived myoblasts which do not differentiate during development, constitute at least partially the adult muscle stem cells, named satellite cells (Gros, Manceau, Thomé, & Marcelle, 2005).

In mature adult muscle, satellite cells are mitotically quiescent, but can be activated in response to injury or disease. Unlike embryonic myogenesis, muscle generation in higher vertebrates requires an extracellular matrix scaffold on the injured tissue for the formation of muscle fibers (Ciciliot & Schiaffino, 2010). Curiously, only amphibians and some fish are capable of regenerating muscle without a supportive and appropriate scaffold tissue (Poss, 2010). Satellite cells follow the same genetic hierarchy regulating embryonic myogenesis and can be identified by the expression of specific markers such as m-cadherin, myocyte nuclear factor MNF, CD34 and Myf5. They also use asymmetric cell divisions for self-maintenance and to generate more committed myogenic progenitors (Kuang, Kuroda, Le Grand, & Rudnicki, 2007; Shinin, Gayraud-Morel, Gomès, & Tajbakhsh, 2006). Indeed, upon activation, satellite cells proliferate, and some of these will differentiate and participate in the regeneration process, while a minor population will reform the reservoir of satellite cells in the muscle (Shi & Garry, 2006) (Figure 9).

3.2. Molecular components of myoblast differentiation

The differentiation of skeletal muscle in vitro is a well-known developmental and differentiation model, characterized by the sequential induction of various members of the MyoD basic helix-loop-helix transcription factor family (Pownall, Gustafsson, & Emerson, 2002). Differentiation studies have largely made use of myoblast cell lines, such as the mouse C2C12 cell line, which is capable of inducible muscle differentiation. Upon removal of mitogens, proliferating mononucleated myoblasts stop dividing and fuse into multinucleate myotubes through the sequential expression and combinatorial association between basic helix-loop-helix transcription factors (Massari & Murre, 2000) and myocyte-specific enhancer-binding protein MEF2 (Naya & Olson, 1999) (Figure 9). Proliferating myoblasts express the helix-loop-helix proteins MyoD and Myf5, which bind to MEF2 and histone deacetylase 1 (HDAC1) and thereby inhibit their activities (McKinsey, Zhang, & Olson, 2002). Upon induction of differentiation, MyoD is derepressed and promotes the expression of another helix-loop-helix protein, myogenin, and the cell-cycle regulators p21, cyclin D3 and Rb. The induction of these key cell-cycle regulators and consequent dephosphorylation

of Rb allow cells to enter into a postmitotic state and differentiation to proceed (Novitch, Spicer, Kim, Cheung, & Lassar, 1999). Myf5 and MyoD are important determinants for the myogenic lineage while myogenin is essential for muscle differentiation. In adult muscle, Myf5 is expressed in quiescent satellite cell, while MyoD expression is induced upon activation and followed by myogenin as differentiation begins (Figure 9). Myogenin induces the expression of contractile proteins such as myosin heavy chain (MHC) and troponin (Beauchamp et al., 2000; Cooper et al., 1999). Other important players of muscle differentiation are proteases such as caspase-3 and calpains whose activity is necessary for early initiation of the process, perhaps to allow cleavage and disassembly of superfluous myoblast proteins and thereby make space for the expression of differentiated cell-specific proteins (Fernando et al., 2002; Liang, Yeh, Forsberg, & Ou, 2006).

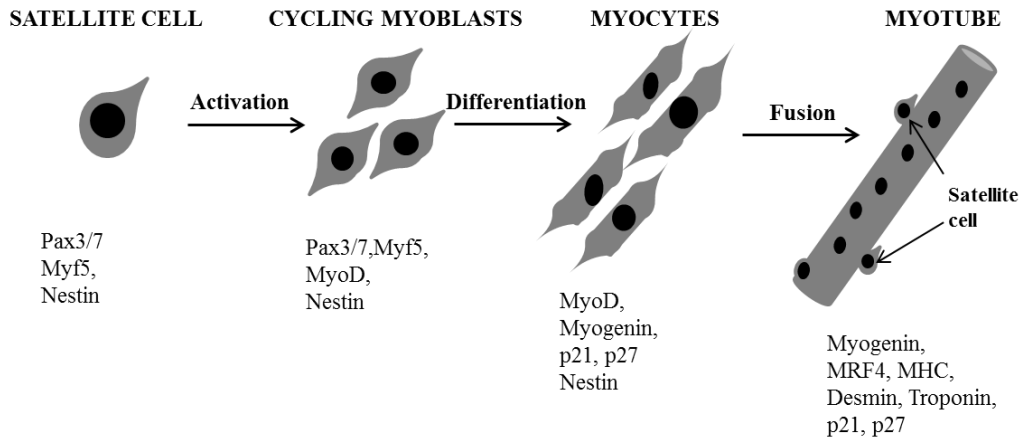

Figure 9. Schematic representation of adult myogenesis. Quiescent skeletal muscle satellite cells can be activated upon injury or stimuli from the associated fiber. Activated satellite cells begin to proliferate and turn into myoblasts, which express the master regulators Pax3/ 7 and the myogenic regulatory factors MyoD and Myf5. The majority of myoblasts will differentiate into myocytes, which stop cycling, lose expression of Pax3/ 7 and Myf5, migrate, adhere and fuse with each other and to other myotubes to form large myotubes with many nuclei. A proportion of the cycling myoblasts will instead reconstitute the reservoir of satellite cells (modified from Le Grand et al., 2007).

3.3. Cdk5/p35 signaling

Cyclin-dependent kinase 5 (Cdk5) is a small serine-threonine kinase which is structurally homologous to the cell cycle Cdks (Lew & Wang, 1995). Although Cdk5 binds to cyclin D and cyclin E (Guidato, McLoughlin, Grierson, & Miller, 1998; Xiong, Zhang, & Beach, 1992), there is no evidence that Cdk5 activity is regulated by these cyclins (Morgan, 1997). Instead, activation of Cdk5 has been shown to be regulated by the cyclin-related proteins,, p35, and p39 (Dhavan & Tsai, 2001). Although Cdk5 is widely expressed in mammalian tissues, its activators are expressed mainly in neurons and during early stages of myogenesis (De Thonel et al., 2010; Lazaro et al., 1997; Sarker & Lee, 2004). Cdk5 and p35 participate in the development of neuronal cells and axonal growth (Paglini et al., 1998; Pigino, Paglini, Ulloa, Avila, & Cáceres, 1997), and are essential for neuronal migration in the cerebral cortex (Chae et al., 1997; Ohshima et al., 1996). In the brain, Cdk5 is not found in dividing cells but is expressed primarily in differentiated neurons. Hence, unlike other Cdks, Cdk5 does not

appear to participate in cell cycling but is rather involved in regulating cell differentiation and nuclear and cytoskeletal processes (reviewed in Nguyen et al., 2002). Indeed, Cdk5 has been shown to be necessary and sufficient for neuronal cell cycle arrest and subsequent differentiation (Ohshima et al., 1996; T. Tanaka et al., 2001). Accordingly, analysis of the cortex of a Cdk5-deficient murine embryo has shown that nestin expression, a well-known marker for proliferative stem cell, remains elevated also at an advanced stage of differentiation and typical differentiation markers such as Map2 are absent (Cicero & Herrup, 2005).

Cdk5 activity is necessary also for muscle differentiation. Lazaro and coworkers showed that Cdk5 activity increases markedly during early stages of myoblast differentiation. During this time, Cdk5 changes localization and moves from the cytoplasm to the nucleus. Overexpression of Cdk5 increases myogenic differentiation while inhibition of Cdk5 activity inhibits myogenesis in C2C12 cells, indicating that Cdk5 acts as a positive modulator of differentiation (Lazaro et al., 1997). Our previous work identified nestin as a target of Cdk5 during myoblast differentiation where it functions as a scaffold for the Cdk5/ p35 signaling complex. Upon activation, Cdk5 phosphorylates nestin at Thr316, thereby increasing the levels of unpolymerized nestin and allowing the reorganization of nestin into parallel fibers (Sahlgren et al., 2003). Our more recent study further clarifies the role of nestin in determining the amount of active Cdk5/ p35, and thereby regulating the differentiation process. Indeed when nestin was overexpressed, it strongly promoted myogenesis, whereas myogenesis was inhibited by nestin downregulation (Pallari et al., 2011).

3.3.1. The Cdk5 activator, p35

p35 is a highly unstable protein with a half-life of 20-30 minutes as determined in rat cortical neurons (Patrick, Zhou, Kwon, Howley, & Tsai, 1998). p35 can be cleaved between residue Phe-98 and Ala-99, resulting in the removal of the N-terminal fragment (amino acids 1-98) and in the formation of the more active and more stable fragment p25 (Uchida et al., 1994) (Patrick et al., 1998). The cleavage of p35 removes the myristoylation signal located in the N-terminal end, and shifts the Cdk5/ p35 complex from a membranous to a cytoplasmic localization (Figure 10) (Kusakawa et al., 2000; Patrick et al., 1999). The p25 fragment can also target Cdk5 to the nucleus where Cdk5 has been shown to phosphorylate and regulate the activity of several transcription factors, such as the pro-survival factor MEF2 (P. D. Smith et al., 2006) and the tumor suppressor p53 (Zhang et al., 2002). Even though the generation of p25 has been generally associated to apoptosis in several neuronal systems (Hamdane et al., 2005; O'Hare et al., 2005; P. D. Smith et al., 2006), it is not clear whether it is the elevated Cdk5-p25 activity or the change in localization and target proteins which mediate cell death.
Calpain was identified as the enzyme responsible for the cleavage of p35 (Kusakawa et al., 2000; M. S. Lee et al., 2000). Indeed, calpain inhibitors reduce or completely abolish conversion of p35 to p25 (M. S. Lee et al., 2000; Patzke & Tsai, 2002). As calpains are calcium-activated proteases involved in hypoxic/ ischemic and other neuronal cell death paradigms, calcium homeostasis is likely to be crucial in the regulation of Cdk5 activity, especially under pathological conditions characterized often by a disrupted calcium homeostasis. It also seems that phosphorylation of p35 at Ser8 and Thr138 by Cdk5, protects p35 by calpain-mediated cleavage and that dephosphorylation of Thr138 is critical to allow p25 formation (Kamei et al., 2007).

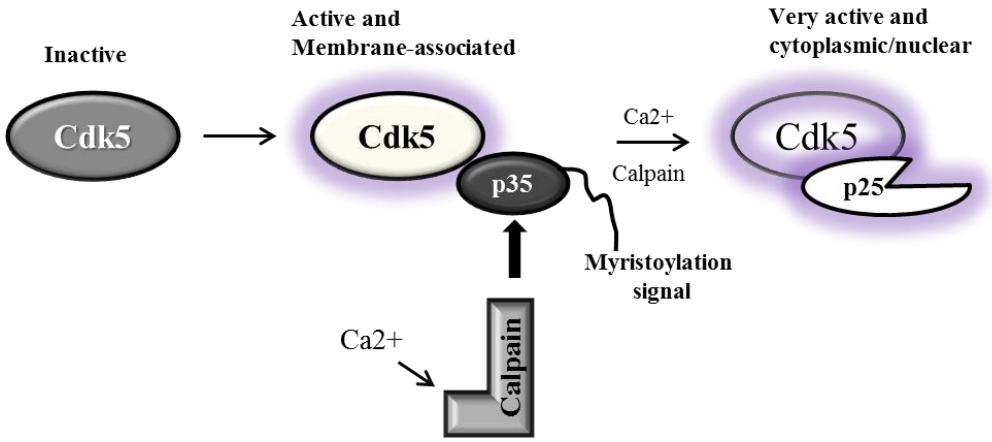

Figure 10. Schematic representation of the mechanism of regulation of Cdk5. Binding of the activator p35 to Cdk5 targets the complex to membraneous fractions of the cell. Upon calpain activation, p35 is cleaved into the more active fragment p25 and loses its myristoylation signal. The Cdk5/ p25 complex is activated and distributes into the cytoplasn and nucleus where it targets different substrates.

3.3.2. Cdk5 targets and functions

As an exception within the Cdk family, Cdk5 does not seem to be involved in cell cycle progression, although it can bind and phosphorylate the Rb protein (Honma, Hosono, Kishimoto, & Hisanaga, 1997). Instead, several other biological functions have been described for Cdk5. Many Cdk5 target proteins are cytoskeletal proteins, kinases involved in cytoskeletal regulation or neuronal migration, cell adhesion molecules involved in membrane cycling and axonal transport, such as neurofilaments, MAP1B, c-Abl, cables, PAK1, Src, Nudel, b-catenin (Kesavapany et al., 2001; Niethammer et al., 2000; Paglini et al., 1998; Sun, Leung, & Liem, 1996; Zukerberg et al., 2000). A common function of these different Cdk5 targets is the regulation of cell morphology and motility, including neuronal migration, axonal outgrowth and axonal transport. Therefore, not surprisingly, the targeted disruption of *Cdk5* induces a prenatally lethal phenotype caused by a CNS dysfunction (Ohshima et al., 1996, 1999). *Cdk5*-deficient mouse embryos show a widespread disruption of cortical layering, again suggesting a role for Cdk5 in neuronal migration. Interestingly and accordingly, double knockout of p35 and of the second major Cdk5 activator p39 result in a phenotype identical to Cdk5-deficient mice (Ko et al., 2001). Some studies also suggested a role for Cdk5 in dopamine signaling and in the regulation of drug addiction upon cocaine administration (Bibb et al., 2001; White & Cooper, 2001). Two other studies showed that NMDA receptors are another target of Cdk5 and that their phosphorylation results in decreased long-term potentiation and regulate neuronal plasticity and cognitive functions (Li et al., 2001; Fischer et al., 2002).

In addition to neuronal morphology and migration, Cdk5 activity has been associated to neuronal cell death, such as in neurons subjected to excito-toxins, β-amyloid, and oxidative stress (Alvarez, Toro, Cáceres, & Maccioni, 1999; Gong et al., 2003; M. S. Lee et al., 2000), as well as in animal models of stroke (J. Wang, Liu, Fu, Wang, & Lu, 2003), Parkinson's disease (P. D. Smith et al., 2003), and amyotrophic lateral sclerosis (Nguyen, Larivière, & Julien,

2001). Importantly, each of these models is thought to involve deregulated Ca^{2+} signaling and display elevated production of the cleaved fragment p25, which is almost non-detectable under physiological conditions (Lee et al, 2000). Indeed, Cdk5 has been shown to promote cell death when its activator p35 is cleaved into the smaller and more active form p25 by calpains (Kusakawa et al., 2000; M. S. Lee et al., 2000; Patrick et al., 1999) (Figure 10). Accordingly expression of Cdk5/ p25 induces apoptosis in primary cortical neurons (Patrick et al., 1999; Gon et al., 2003). Cdk5/ p25 has been shown to increase the levels of phospho-Tau while a dominant -negative Cdk5/ p25 abolishes Tau phosphorylation in cortical neurons (Ahlijanian et al., 2000; Patrick et al., 1999). Accordingly, overexpression of p25 in transgenic mice results in neurodegenerative phenotype including formation of paired helical filaments, Tau aggregation and neuronal loss similar to that observed in Alzheimer's disease (Pei et al., 1999; Patrick et al., 1999; Tseng at al., 2002). Also, p25 formation enables the shuttling of Cdk5 into the nucleus where Cdk5 phosphorylates and inhibits the pro-survival transcription factor MEF2. Phosphorylation of MEF2 has been demonstrated to make cortical neurons more sensitive to oxidative stress (Gong et al., 2003; O'Hare et al., 2005; P. D. Smith et al., 2006). Other nuclear targets of Cdk5 are the survival factors p53 and Rb. Excessive Cdk5 activity can indeed contribute to neuronal cell death via phosphorylation of p53 (Zhang, Krishnamurthy, & Johnson, 2002) and Rb (Hamdane et al., 2005). In fact, Cdk5-mediated phosphorylation of p53 disrupts the binding to the E3 ubiquitin ligase Hdm2, thereby increasing p53 protein levels (Lee et al., 2007). Furthermore, Cdk5-mediated phosphorylation promotes the acetylation and transcriptional activivation of p53, and consequently the induction of pro-apoptotic genes (Lee et al., 2007). Despite the large number of data linking the induction of apoptosis to increased Cdk5 activity and p25 accumulation, the conditions which turn Cdk5 into an active effector of neuronal death remain unclear and are mainly attributed to the cleavage of the regulator p35.

3.4. Atypical PKCs

The protein kinase C (PKC) family is formed by 10 different lipid-dependent serine/ threonine kinases (PKCα, βI, βII, γ, δ, ε, η, θ, ξ and λ/ ι) which have been implicated in a wide range of cellular processes, such as cell growth, transcription, immune responses, membrane structure regulation, learning and memory (Breitkreutz, Braiman-Wiksman, Daum, Denning, & Tennenbaum, 2007; W. S. Liu & Heckman, 1998). PKCs exhibit distinct tissue distribution: PKCα, β, δ, ε and ξ are ubiquitously expressed, PKCγ is restricted to the central nervous system and spinal cord, PKCη is strongly expressed in the skin and in the lung, PKCθ in the skeletal muscle tissue, and PKCη in numerous tissues and strongly in the thymus and lung.

The structure of PKC isozymes includes conserved domains referred to as C1-C4, interrupted by variable regions V1-V5 of unknown function (Figure 10). The C1 region, located close to the N-terminal, contains two main motifs. One resembles the consensus sequence found in the phosphorylation sites of major PKC substrates, except for having an alanine residue in place of a serine or threonine as a phospho-acceptor and thereby blocking the substrate-binding site of the kinase. This motif serves as an autoregulatory domain, referred to as pseudo-substrate (PS). The second motif of the C1 domain contains two cysteine-rich sequences, important to mediate binding to diacylglycerol (DAG) and phorbol esters. The atypical PKCs (aPKC) differ in this region because they possess only half of the C1 domain and therefore, cannot be activated by C1 ligands such as DAG and phorbol esters (Figure 11). The C2 domain contains many acidic amino acids which are thought to participate in binding to Ca2+. The C-terminal regions, C3-V5, constitute the catalytic domain. The C3 region contains the ATP binding sequence similar to the one present in other protein kinases.

Finally, the C4 domain contains the substrate-binding region and the phosphate-transfer site (Figure 11).

According to their structure and mode of activation, the PKC superfamily has been subdivided into three subgroups: 1) the conventional PKC isoforms (cPKC), including PKCα, PKCβ and PKCγ that are activated by diacylglycerol (DAG) and Ca2+; 2) the novel PKC isoforms (nPKC) (PKCδ, ε, η, θ) that are only sensitive to DAG but not to Ca2+; 3) the atypical PKC isoforms (aPKC), the PKCξ and PKCλ/ι, that are neither sensitive to DAG nor to Ca2+ (Parker & Murray-Rust, 2004) (Figure 11).
All three subgroups are regulated by phosphatidylserine, 3'-phospho-inositide-dependent kinase-1(PDK1), and auto-phosphorylation (Newton, 2001).

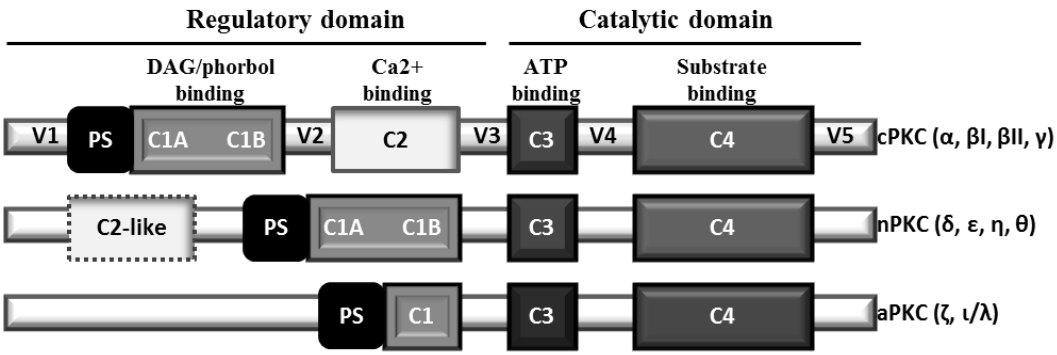

Figure 11. Structural characteristics of the PKC family
Each PKC contains a highly homologous C-terminal catalytic domain and an N-terminal regulatory domain. The DAG/ phorbol-ester-binding C1 domain, which is constituted of two repeated cysteine-rich zinc-finger motifs (C1A and C1B), is functional in the conventional PKCs (cPKC) and in the novel PKCs (nPKC) but not in the atypical PKC (aPKC). The C2 domain mediates Ca^{2+} binding in cPKCs, but differences in the key residues abrogate this function in nPKCs. aPKCs have single modified C1 domain and hence cannot bind to Ca2+. C3 and C4 form the ATP- and substrate-binding regions of the kinase core. The autoinhibitory pseudosubstrate sequence (PS) interacts directly with the substrate-binding cavity in the catalytic domain, thereby sterically blocking access of substrates to the active site.

The atypical PKCs are involved in a wide range of physiological processes including mitogenesis, protein synthesis, cell survival and transcriptional regulation. The two sub-family members, PKCξ and PKCλ/ι, are highly related, with a 72% overall amino acid identity (Nishizuka, 1995). The main sequence divergence is in the last 60-residues segment of the N-terminal regulatory domain. PKCξ deficient mice are viable but show impaired NF-κB signaling and immune response (Leitges et al., 2001; P. Martin et al., 2002). Instead, PKCλ-deficient mice die at an early embryonic stage (Akimoto *et al*, unpublished results). The differential contribution of these two isotypes might be due to their different expression levels and tissue distribution but isoform-specific roles have been difficult to identify due to the high sequence similarity between PKCξ and PKCλ. Indeed all aPKC binding proteins

reported so far bind to both aPKC isoforms or at least binding exclusivity to one isoform has not been demonstrated.

3.4.1. PKCξ activation mechanism

The mechanism of activation of aPKC primarily consists of two events: the release of the autoinhibitory domain and the phosphorylation of the kinase domain (Newton, 2001). The PKC C1 domain contains an autoinhibitory pseudosubstrate sequence that keeps the enzyme in an inactive form by masking one or more phosphorylation site(s). Interaction with membrane lipids and metabolites, such as phosphatidylserine and DAG, induce the release of the pseudosubstrate from the active site in conventional and novel PKCs, while interaction with lipid components, such as arachidonic acid and ceramide, promotes the release of the pseudosubstrate in the atypical PKCs (W. S. Liu & Heckman, 1998). After the release of the pseudosubstrate, PDK-1 phosphorylates the activation loop of PKC members (Balendran, Hare, Kieloch, Williams, & Alessi, 2000; Le Good et al., 1998). PDK-1 phosphorylates Thr410, which is essential for PKCξ activation. Following Thr410 phosphorylation, Thr560 is targeted by PKCξ autophosphorylation, leading to a complete activation of the kinase (Le Good et al., 1998; Standaert, Bandyopadhyay, Kanoh, Sajan, & Farese, 2001). PKCξ is activated by several lipid groups, including phosphatidic acid (Nakanishi & Exton, 1992), phosphatidylinositol 3,4,5-triphosphate (Nakanishi, Brewer, & Exton, 1993), arachidonic acid and ceramide (Müller et al., 1995). Consistently, aPKCs are strongly implicated as downstream effectors of PI 3-kinase (Chou et al., 1998; Sontag, Sontag, & Garcia, 1997; Takeda et al., 1999). Protein-protein interactions play a major role in restricting the localization and the activation of aPKC and are therefore described in more detail in the following paragraph (Moscat et al., 2000).

3.4.2. Regulation of aPKC activity through protein-protein interaction

A key event in PKCξ activation and in the engaged signaling pathway relies on the association of PKCξ with other signaling molecules and scaffold proteins that mediate transport and shuttling to distinct subcellular sites or act as anchors to allow the formation of oligomeric signaling complexes. Here below are listed the most well-known aPKC interacting domains and the respective binding molecule and the activated pathway:

- PB1 domain

The PB1 domain is a conserved structural module of about 80 amino acids that mediates protein-protein interaction between signaling molecules (T. Ito, Matsui, Ago, Ota, & Sumimoto, 2001). The PB1 domain of aPKC serves to locate aPKCs into distinct subcellular regions and signaling complexes and is located in the V1 region which is conserved among the aPKCs but shows no similarity to other PKC isoforms (T. Ito et al., 2001; D. Lin et al., 2000). For example, p62/ ZIP, an SH2-interacting protein, acts as a scaffold to recruit aPKC into different signaling complexes, such as the TNFα receptor complex or the IL-1 complex, where aPKC can phosphorylate and regulate IKKβ (Sanz, Diaz-Meco, Nakano, & Moscat, 2000; Sanz, Sanchez, Lallena, Diaz-Meco, & Moscat, 1999). P62/ ZIP can promote the recruitment of aPKC also to the NGF receptor complex where it mediates cell survival (Wooten, Seibenhener, et al., 2001). aPKCs can also utilize their PB1 domain to interact with the partitioning proteins PAR-3 and -6, determinants of asymmetric cell division and polarized cell growth (Tabuse et al., 1998; Hung and Kemphues, 1999). PAR-6 targets aPKC to the small GTPases of the Rho family, Rac1 and Cdc42, in a GTP-dependent manner. Binding of PAR-6 only usually causes the suppression of PKC kinase activity, while association of Rac1/ Cdc42 to PAR-6 relieves the suppression of aPKC (Joberty, Petersen, Gao, & Macara, 2000; D. Lin et al., 2000; Qiu, Abo, & Steven Martin, 2000; Suzuki et al., 2001). Therefore, Cdc42 functions as an activator of the PAR-6/ aPKC complex. The PAR-

6/ aPKC/ Cdc42 complex has been shown also to interact with the glycogen synthase kinase-3β (GSK-3β) and thereby to promote the polarization of the centrosome and the direction of cell protrusion (Etienne-Manneville & Hall, 2003). In this context, the PAR-6/ aPKC/ Cdc42 complex phosphorylates and inactivates GSK-3β at the edge of a migrating cell and allows the establishment of cell polarity. PKCξ can interact via its PB1 domain also with MEK5, functioning as an adapter in the MEK5/ ERK5 pathway, a MAPK cascade critically involved in mitogenic activation by EGF (Diaz-Meco & Moscat, 2001). PKCξ in this case is essential to promote ERK5 activity and interestingly it does so by functioning as an adapter independent of its enzymatic activity.

- Cysteine-rich domain

The single cysteine-rich domain located in the C1 conserved region of aPKC interacts with PAR-4 (prostate androgen response-4), whose expression is induced during apoptosis. PAR-4 binds to the single cysteine-rich sequence of aPKC and thereby inhibits the aPKC kinase activity independently of lipid binding. This results in the suppression of the pro-survival role of aPKC and enhancement of apoptosis (Díaz-Meco et al., 1996).

- PXXP motif

Wooten and coworkers have shown that Src uses its SH-3 domain to bind to PXXP-rich sequences located between the C1 and the PB1 domain of aPKCs (Wooten, Vandenplas, Seibenhener, Geetha, & Diaz-Meco, 2001). This association is enhanced by NGF treatment and promotes the formation of a complex containing the NGF receptor TrkA, Src and PKCι. PKCι interacts with active but not with inactive Src which, in turn, phosphorylate PKCι at different sites. It has been proposed that phosphorylation of specific aPKC tyrosine residues by Src may dictate the type of interaction and the component of the binding proteins, and thereby the type of engaged signaling pathway.

3.4.3. PKCξ targets and functions

An increasing number of studies indicate that the aPKC isoforms are critically involved in a number of important cellular functions, such as the regulation of cell proliferation and survival, and neuronal and leukemic differentiation. Here below are listed the most well-known functions.

Upon activation by ceramide or by NGF treatment, PKCξ translocates from the cytoplasm to the nucleus and regulates both differentiation and cell survival of neuronal cells (Calcerrada et al., 2002; White et al., 2002; Wooten et al., 1997). Upon nuclear entry, PKCξ locates to the inner nuclear matrix where it binds chromatin and plays critical roles in regulation of transcription, ribosomal RNA biosynthesis and shuttling of mRNA. Several nuclear targets of PKCξ have been identified including SP1 (Pal, Claffey, Cohen, & Mukhopadhyay, 1998), NF-κB (A. G. Martin, San-Antonio, & Fresno, 2001), nucleolin (Zhou, Seibenhener, & Wooten, 1997), and hnRNP A1 (Municio, Lozano, Sánchez, Moscat, & Diaz-Meco, 1995). PKCξ mediates cytoprotection against drug- or UV-induced apoptosis (Huang, Ma, Bowden, & Dong, 1996; Jamieson, Carpenter, Biden, & Fields, 1999). In these cases, ceramide, produced under various apoptotic stimuli, may work as a second messenger to activate PKCξ (Lozano et al., 1994). Furthermore, PKCξ has been reported to be a substrate for several caspases (Smith et al., 2000). Interestingly, the other atypical PKC, PKCλ, is resistant to caspase-mediated cleavage as it does not have a caspase processing site. Caspase -3, -6,-7, and -8 cleave PKCξ in the hinge region between the regulatory and catalytic domains and generate a free kinase domain fragment (Lucinda Smith, Wang, & Smith, 2003). Also, PKCξ has been reported to regulate the NF-κB pathway by phosphorylating IκB and p64 subunit (Rel A) at Ser311 residue and, thereby, mediating the anti-apoptotic role of NF-κB (Duran, Diaz-Meco, & Moscat, 2003; Savkovic, Koutsouris, & Hecht, 2003). PKCξ is also

indispensable for the establishment of epithelial cell polarity. The polarity protein PAR-3 was identified as an interacting protein of PKCξ (Izumi et al., 1998). PAR-3 binds to a transmembrane component of the tight junctions (JAM) and recruits the PKCξ/ PAR-6 complex to it (Nagai-Tamai, Mizuno, Hirose, Suzuki, & Ohno, 2002). PKCξ is therefore necessary to establish the asymmetric submembranous structures which enable the subsequent organization of cell polarity. Defect in one of the protein complex causes the mislocalization of the other proteins and the disruption of cell polarity. The downstream targets of aPKCs for the establishment of epithelial cell polarity are still unknown (Nagai-Tamai et al., 2002). Interestingly, aPKCs are present only in multicellular organisms, for which establishment of cell polarity is essential, suggesting that the appearance of aPKCs might have been a critical event for the development of multicellular organisms (Suzuki et al., 2001). PKCξ also participates in protein transport by recruitment to the pre-Golgi compartment through association with the small GTPase Rab2. Inhibition of PKCξ blocks Rab2-mediated reverse translocation and results in an accumulation of swollen vesicles at the pre-Golgi compartment, suggesting that PKCξ modulates recycling cargos (Tisdale, Wang, Silver, & Artalejo, 2003). Lastly several studies have demonstrated that active PKCξ, the processed catalytic fragment (PKMξ), is necessary and sufficient for maintenance of long term potentiation (LTP) and memory but its downstream targets remain to be identified (Drier et al., 2002; Ling et al., 2002).

OUTLINE OF THE STUDY

A timely area of research is the crosstalk between signals that regulate survival, cell stress, differentiation, and programmed cell death. Often the same signaling factors can be involved in regulating several processes, depending on the context at which they are activated. In this regard, I was especially interested in AATF and Cdk5, as both of these signaling molecules form multitasking complexes, in a way like operational joysticks on the road to cellular fate.

The starting point of this project was based on our and other previous works which identified both Cdk5/p35 and PKCξ as positive and independent regulators of the differentiation process. The goal of my study was to investigate whether these two kinases were regulating the same signalling cascade pathway and whether one was the upstream regulator of the other.
Furthermore, I was intrigued by a novel and unexplored transcription factor involved in the regulation of stress responses and apoptosis, AATF, which was shown to have both the property of promoting cell proliferation and inducing cell cycle arrest. During the course of this work, I cloned several AATF isoforms from different murine tissues and investigated the role of AATF in response to apoptotic insults such as UV irradiation. In summary the aims of this thesis were:

- To characterize the role and target of AATF upon an apoptotic insult.
- To identify upstream regulators of the Cdk5/p35 complex during muscle differentiation.

EXPERIMENTAL PROCEDURES

1. Plasmid constructs (I-III)

Plasmids encoding c-Jun-HA, Gal4-c-Jun, Gal4-c-JunAA, and Gal4-c-JunΔ have been described earlier (Ferraris *et al.*, 2012; Weiss *et al.*, 2003; Westermarck *et al.*, 2002). p35 and Cdk5 cloned into pcDNA3.1 His vectors were kindly provided by Dr. Harish Pant (National Institutes of Health, Bethesda, USA). Myc-tagged p35 was purchased from Addgene. Pint mutation to this vector was made using QuickChange site-directed mutagenesis kit (Stratagene) and verified by sequencing. The Flag-tagged PKCζ and PKCι were kindly provided by Dr. John Blenis (Harvard Medical School, Boston, USA). The following procedure was followed to clone AATF into EGFP vector. RNA was isolated following standard techniques (Trizol) from murine muscle, brain, lung tissues and retro-transcribed into cDNA using random primers and RT according to the manufacturer's instructions. The following primer pairs were used in order to amplify and clone AATF isoform 1, 2 and 3 from cDNA into EcoRI and KpnI sites in-frame with the C-terminal EGFP tag in EGFP-C1 vector or with the N-terminal EGFP tag in EGFP-N3 vactor.

Forward primer for AATF1 and 2 (cloning into EGFP-C1):
5'GCGAATTCTATGGCGGCGCCGCAGCCCTTG3';
Reverse primer:
5'CCGGTACCTCACTTCCCACGGTCTGCATC3';

Forward primer for AATF1 and 2 (cloning into EGFP-N3):
5'GCGAATTCTATGGCGGCGCCGCAGCCCTTG3';
Reverse primer for AATF1 and 2:
5´GACGGTACCCTTCCCACGGTCTGCATCAGG3´;

Forward primer for AATF3 (cloning into EGFP-C1):
5'GCGAATTCTATGGCGGCGCCGCAGCCCTTG3';
Reverse primer for AATF3:
5´CCGGTACCGAGCTGGTGGTAAAAGTCATC3´

2. Cell culture (I-III)

All cell lines were maintained in a humified 5% CO2 atmosphere at 37°C. HEK293 and Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma), containing 10% fetal bovine serum (FBS), 2mM L-glutamine, and antibiotics (penicillin and streptomycin). c-Jun-KO MEFs and wt MEFs were cultured in DMEM containing 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate.

C2C12 cell line is a subclone from a mouse myoblast cell line isolated from the thigh muscle of an adult mouse (Yaffe and Saxel, 1977). This cells line was maintained in DMEM supplemented with 10% FBS, 2mM Glutamine, and antibiotics (growth medium). Differentiation of C2C12 myoblasts was induced by replacing growth medium with DMEM supplemented with 1% FBS, 2mM L-Glutamine, and antibiotics (differentiation medium).

3. Experimental treatments (I-III)

The inhibition of PKCξ activity was achieved by addition to the growth or differentiation medium of 15-20 μM PS peptide inhibitor (PS; Myr-SIYRRGARRWRKL; MilleGen Prologue Biotech). Scramble peptide (Scr-P;Myr-RLYRKRIWRSAGR) was used as a control to exclude unspecific effects due to peptide treatments. Calphostin C was used to specifically inhibit the cPKCs and nPKCs. To inhibit Cdk5 activity Roscovitine was added to the medium at 10 μM.

JNK inhibitor SP-600125 was used at 20 μM. Calpain activity was induced by adding 10 μM of calcium ionophore A23187 (Calbiochem). The inhibition of calpain activity was obtained with 15 μM calpain inhibitor III (Calbiochem).

For UV irradiation, cells were left to grow until they reached 80% confluency. Medium was removed and cells were rinsed once with PBS. Cells were than exposed to UVC irradiation, with an intensity of 15-20 μJ for MEF cells and 100-120 μJ for HEK293 cells. Immediately after irradiation, PBS was replaced with warm growth medium and cells were further incubated for further analysis.

4. Isolation and culture of primary myoblasts (III)

Primary myoblasts cultures were established from the limb of newborn mice. Muscle tissue was minced and enzymatically digested by incubation in 0.2% type XI collagenase (Roche Diagnostics) and 0.1% trypsin at 37°C for 45 minutes. The resulting slurry was filtered to remove large pieces of tissue and rinsed with growth medium (Hams F-10 [Sigma] supplemented with 15% FBS, 2 M L-glutamine, antibiotics, and 2.5 ng/ ml fibroblast growth factor-β [Promega]). Cells were centrifuged at 1000 x g for 5 min, resuspended in growth medium, and plated into tissue culture dishes. When cells reached 80% of confluence, differentiation was induced by replacing growth medium with differentiation medium (DMEM supplemented with 1% FBS, 2mM L-Glutamine, and antibiotics).

5. Transfections (I-III)

Hela and HEK293 cells were transfected by electroporation. For this purpose, approximately 5X10^6 cells were collected, centrifuged and resuspended in 0.4 ml of OptiMEM (Gibco). After addition of 20-40 μg plasmid DNA, cells were transferred in 0.4 cm electroporation cuvettes (BTX) and subjected to single electric pulse (220 V, 975 μF) using a Bio-Rad Gene Pulser electroporator. Electroporated cells were than resuspended in growth medium and plated. Expression of transfected plasmids was assessed after 24-48 hours.

C2C12 and mouse primary myoblasts were transfected using the Lipofectamine plus reagent (Life Technologies) according to manufacturer's instructions.
The downregulation of PKCξ was achieved by transfecting cells with 20 or 80 pmol of PKCξ siRNA pool (Santa Cruz Biotechnology) using Lipofectamine plus reagent. AATF was downregulated using AATF siRNA or scrambled siRNA (Qiagen), transfected with Lipofectamine RNAiMAX reagent (Life Technologies) according to manufacturer's instructions.

6. Western blotting (I-III)

Cell extracts were prepared by lysis in Laemmli buffer, subjected to SDS-polyacylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane (Protran nictrocellulose, Schleicher & Schuell) using semidry or wet transfer apparatus (Bio-Rad, GE Healthcare). Membranes were blocked with 5% non-fat milk in PBS containing 0.3% Tween-20 for 1 h at RT. Western blotting were performed using antibodies against AATF (used at 1:500; Cell Signaling Technology), c-Jun (1:500; Santa Cruz Biotechnology), phS63 c-Jun (1:1000; Cell Signaling Technology), GFP (1:5000; JL-8, Living Colors), nestin (BD Pharmigen), p35 (1:1000; C-19 and N-20, Santa Cruz Biotechnology), Cdk5 (1:1000; DC34, Biosource Invitrogen and C-8, Santa Cruz Biotechnology), PKCζ (1:500; Santa Cruz Biotechnology), poly(ADP-ribose)polymerase (1:1000; PARP; Sigma), p21 (1:1000; C-19, Santa Cruz Biotechnology), myogenin (1:500; M-225, Santa Cruz Biotechnology), troponin (1:500; JLT-12, Sigma), MHC (1:1000; H-300, Santa Cruz Biotechnology), desmin (1:500; RD301, Santa Cruz Biotechnology), calpain 1 (1:1000; Cell Signaling Technology), calpain 3 (1:500;

Abcam), RhoA (1:200; Santa Cruz Biotechnology), 14-3-3τ (1:1000; Santa Cruz Biotechnology), HSC70 (1:10,000; SPA-815, Stressgen), actin (1:1000; AC-40, Sigma). After washed with 0.3% Tween-20 in PBS, membranes were probed with horseradish peroxidase-conjugated secondary antibodies (used at 1:10,000; Promega, Southern Biotechnology, and GE Healthcare). Proteins were visualized using ECL detection kit (GE Healthcare).

7. Co-immunoprecipitation analyses (I-III)

Cells were harvested, suspended in lysis buffer (50 mM HEPES, pH 7,4, 140 mM NaCl, 5 mM EDTA, 0.4% NP40, 0.1% Triton, 10 mM pyrophosphate, 1 mM dithiothreitol [DTT], 5 mM sodium orthovanadate, and a protease inhibitor cocktail [Roche Diagnostics, Basel, Switzerland]) and kept on ice for 20 min, followed by centrifugation at 10,000 × *g* for 10 min at 4°C. For AATF or c-Jun immunoprecipitation, lysates were first sonicated and centrifuged at 13.000 g for 10 min at 4°C. Supernatant protein concentration was measured by Bradford assay. 800 µg of protein lysate was used for immunoprecipitation with the indicated antibodies under rotation overnight at 4°C. Immuno-complexes were captured with 50% slurry of protein A-sepharose at 4°C on a rotamix for 1 h. the beads were centrifuged and washed 4 times with 20 mM HEPES (pH 7.4), 2 mM EDTA, 100 mM NaCl, 0.4% NP40, and 1 mM DTT and finally resuspended in Laemmli sample buffer. Immunoprecipitated proteins were eluted from the beads by boiling for 5 minutes in Laemmli buffer, run on a SDS-PAGE and detected by western blotting.

8. Kinase activity assays (III)

Cdk5 or PKCζ were immunoprecipitated from differentiating myoblasts. Cells were lysed in 50 mM HEPES (pH 7,4), 140 mM NaCl, 5mM MgCl2, 5 mM EGTA, 0.4% NP-40, 10 mM pyrophosphate, 1 mM DTT, 5 mM sodium orthovanadate, and a protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland). Lysates were centrifuged at 10.000 g for 10 min at 4°C, supernatants were collected and protein concentration was measured by Bradford assay. 800 µg of protein per lysates was precleared with protein G-sepharose prior to immunoprecipitation with anti-PKCζ or anti-Cdk5 antibodies on a rotator at 4°C overnight. After 1 h incubation with protein G-sepharose beads, the immunoprecipitates were collected by centrifugation and washed 4 times in 50 mM HEPES (pH 7,4), 125 mM NaCl, 5mM MgCl2, 5 mM EDTA, 0.2% NP-40, 1 mM (DTT), and twice in 50 mM HEPES (pH 7,4), 25 mM NaCl, 0, 1 mM DTT. For PKCζ activity beads were resuspended in PKCζ kinase buffer (50 mM Hepes [pH 7.4], 1mM EDTA, 1mM DTT, 10 mM MgCl2) and for Cdk5 activity in Cdk5 kinase buffer (50 mM Tris-HCl [pH 7.4], 5mM MgCl2, 1mM EDTA, 1 mM DTT). The resuspended beads were incubated with histone1 (3µg), 200 µM ATP and 10 µCi of γ-32P ATP for 30 min at 32°C (PKCζ) or at 25°C (Cdk5) and stopped by addition of Laemmli buffer and boiling for 5 minutes. Samples were run on a SDS-PAGE and subjected to autoradiography.

9. Calpain assays (III)

The brains of newborn mice were isolated and homogenized in lysis buffer (20 mM Hepes pH 7.4, 5 mM KCl, 1.5 mM MgCl2, 1 mM DTT, 1 mM EGTA) using a Teflon grinder (Sato *et al*., 2007). The homogenates were centrifuged at 13000 x *g* for 25 min at 4 °C. Pellets were resuspended in 100 µl of lysis buffer, sonicated, and centrifuged again at the same conditions. The supernatants were divided into control and treated sample. Calpain activity was induced by adding CaCl2 at a final concentration of 4 mM and incubating for 1 h at 37°C. The reaction was stopped by adding 10 mM EGTA and an equal volume of Laemmli sample buffer. The cleavage of p35 was assessed by western blotting.

10. Luciferase reporter assay (I-II)

24 h after transfection, cells were harvested and lysed in Passive lysis buffer (Promega) according to manufacturer´s instructions. Cell lysates were centrifuged (15,000 x *g* for 2min) and the supernatants were collected. The firefly luciferase activity produced by the pf2-luc plasmid (Promega) was measured using a Luminoskan Luminometer (Labsystems) using luciferase assay reagent (Promega) as a substrate. The luciferase activity was normalized using RSV-βgalactosidase as an internal control by incubating cell lysates in 100 mM phosphate buffer (pH 7.0) with ONPG (0.670 mg/ ml), MgCl2 (1 mM), β-mercaptoethanol (45 mM) at 37°C and determining the absorbance at 420 nm.

11. Immunofluorescence labeling (I-III)

Transiently transfected cells were plated and grown on coverslips for a minimum of 24 h. Cells were washed with PBS, fixed with 3% parafolmaldehyde (PFA) for 10 minutes at room temperature (RT), and washed 3 times with PBS. Than fixed cells were permeabilized for 10 min in 0.3% Triton X-100 in PBS, and incubated for 1 h in blocking solution (1% bovine serum albumin [BSA] in PBS). Primary antibodies were used at a 1:500 diluition in 5% BSA in PBS for 1 h at RT. After three washes with PBS, cells were incubated with secondary antibodies, Alexa 488 or 568 (Molecular Probes) at a diluition of 1 : 700 for 1 h at RT, washed and mounted on slides in Vectashield containing DAPI (4´, 6´-diamino-2-phenyilindole, Vector laboratories). Cells were analyzed visualized using a Zeiss 510 META laser scanning confocal microscope.

12. Detection of apoptosis (I)

Measurement of apoptosis was detected by flow cytometry counting of double stained cells for Annexing V-APC and propidium iodide, collecting both floating and adherent cells. Cells grown on a 12 wells plate were washed with PBS, resuspended in 350 µl of annexin V binding buffer (2.5 mM HEPES [pH 7.4], 35 mM NaCl, 0.6 mM CaCl2) containing 4 µl/ ml human recombinant Annexin V-APC (BD PharMingen) and 5 µg/ ml propidium iodide (Sigma-Aldrich). After 10 min incubation on ice, samples were directly analyzed on a FACSCalibur flow cytometer (BD PharMingen), by gating GFP-positive cells.

For detection of active caspase-3, cells were washed once in PBS and resuspended in PBS for labeling with PE-conjugated monoclonal active caspase-3 antibody according to manufacturer´s instructions. Counting of the number of GFP-positive cells stained for active caspase- was performed on a FACSCalibur flow cytometer (BD PharMingen).

13. Chip assay

2 X 15 cm2 plates of transfected HEK293 cells were cross-linked with 1 % formaldehide followed by quenching with 125 mM glycine. Pelleted cells were washed twice in PBS and lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1 and a protease inhibitor cocktail). DNA was fragmented by sonication with a Bioruptor system (Diagenode), samples were diluited in immunoprecipitation buffer (20 mM Tris-HCl pH 8.1, 150 mM NaCl, 1% Triton-X, protease inhibitor cocktail), than precleared with protein G-Sepharose beads (GE Healthcare) at 4˚C. The IP was performed at 4˚C overnight using c-Jun antibody (Cell Signaling), Acetylated Histone-4 as positive control, normal rabbit serum (IgG) as negative control. Immunocomplexes were precipitated with protein G beads for 3 hours at 4˚C, than washed 3 times in 0.1% SDS, 1% Triton-X, 2mM EDTA, 20 mM Tris-HCl, 150 mM NaCl, 3 times in differs in 0.1% SDS, 1% Triton-X, 2mM EDTA, 20 mM Tris-HCl, 500 mM NaCl N, twice in 20 mM Tris-HCl, 2mM EDTA, 10% Glycerol. Cross-links were reversed by incubation overnight at 65˚C, and than DNA was purified with phenol-clorophorm and ethanol precipitated. The input lanes represent 1% of the material used for the

immunoprecipitation. PCR analysis was performed using Taq polymerase (Fermentas). The following primers were used:
FasL promoter:
Forward: 5'TGTGTGTGGGGAAGAGATGA3';
Reverse: 5'AAATGGCTCTGAGGGGAGAG3';
Tnfα promoter:
Forward: 5'GTCTCCGGGTCAGAATGAAA3';
Reverse: 5'CTAGAACTGGGAGGGGCTTC3'.

RESULTS AND DISCUSSION

1. Role of AATF in UV-induced stress

1.1 Cloning of AATF

We decided to clone the three different murine isoforms of AATF into EGFP-C1 and EGFP-N3 vector in order to construct an AATF-EGFP fusion protein in which the fluorescent GFP tag would allow an easy visualization of the subcellular localization of AATF. In order to maximize the chance of cloning all known AATF isoforms, we amplified the corresponding cDNAs from three different murine tissues, brain, lung and testis described to express high amounts of AATF. AATF1 cDNA was amplified and cloned from brain tissue, AATF2 cDNA from lung and AATF3 from testis. The amplified AATF cDNAs were cloned into EGFP-C1 and EGFP-N3 vectors. The generated plasmids were digested with EcoRI and KpnI to verify the insertion of a correct size fragment encoding for AATF 1, 2 and 3 of respectively 1581, 1374 and 1239 bp (Figure 1A). Plasmids containing the correct size fragments were sequenced and transfected into Cos7 cells to confirm that the fusion protein AATF-GFP was in-frame and correctly expressed. Cell lysates from the AATF isoforms transfected cells were loaded on a gel for western blot analysis with GFP antibody and confirmed the generation of a fusion protein AATF1-GFP of approximately 95 kDa (Figure 1B and C). Cells transfected with the same AATF constructs were also fixed and mounted on a microscopy glass to visualize the cellular localization of the generated fusion protein. The all three AATF isoforms fused to GFP were able to change the localization of GFP from the cytoplasm to the nucleoli (Figure 1D).

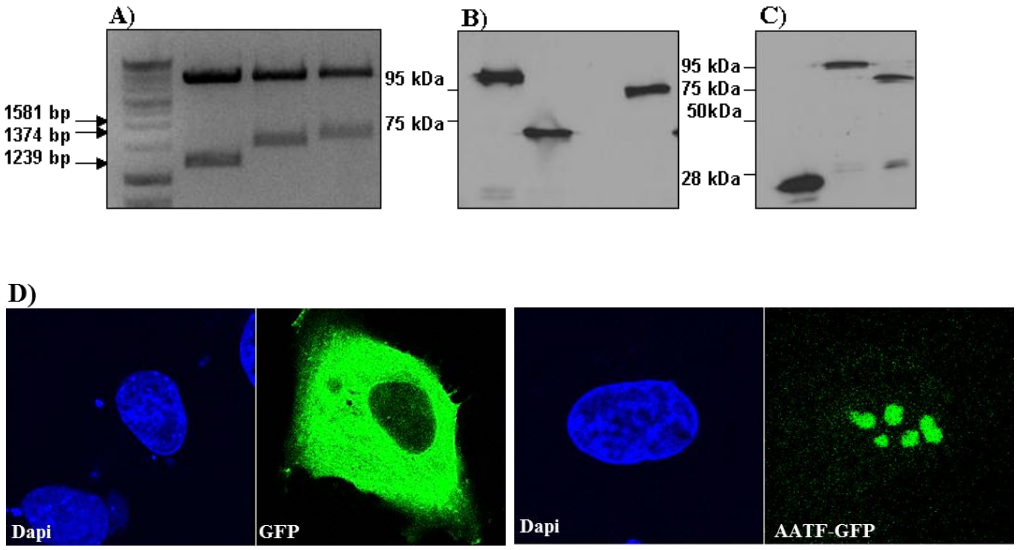

Figure 1. Cloning of the AATF isoforms 1, 2 and 3 into EGFP vector. (A) Amplified AATF was cloned into EGFP vector. Plasmids were screened by restriction digestion with EcoRI and KpnI to verify the insertion of a fragment of 1581 bp corresponding to AATF isoform 1 (AATF1), 1374 bp corresponding to AATF isoform 2 (AATF2) and 1239 bp corresponding to AATF isoform 3 (AATF3). The digested plasmids loaded on an agarose gel show the successful cloning of inserts of the expected size. **(B, C)** The generated constructs AATF1-GFP, AATF2-GFP and AATF3-GFP were transfected into

Cos7 cells to verify that the AATF isoforms cDNAs were in-frame with the EGFP coding sequence and expressed as a fused protein AATF-GFP. Lysates of transfected cells were loaded on a gel for western blotting against GFP. In B) in order from left to right AATF1-GFP, AATF3-GFP, empty, AATF2-GFP, in C) GFP vector, AATF1-GFP, AATF2-GFP**. D**) Cos7 cells were transfected with AATF-GFP or GFP vector, fixed, stained with Dapi to visualize the nucleus and mounted on a microscopy glass. Confocal microscopy images show a cytoplasmic distribution of the empty vector GFP and a nucleolar localization for the fusion construct AATF-GFP.

1.2 AATF expression level determines sensitivity to UV-induced apoptosis

To investigate whether AATF expression level could influence the induction of apoptosis upon UV irradiation, we compared the apoptotic rate of mouse embryonic fibroblasts (MEF) or HEK293 cells overexpressing AATF-GFP or GFP empty vector. For all experiments described in this thesis if not stated otherwise, we used the full length AATF 1 isoform cloned into EGFP-C1 vector as previously described. One day after transfection, cells were exposed to UVC radiation (20 µJ for MEFs, 100 µJ for HEK293). Immediately after irradiation cells were further incubated in new fresh medium and collected for analysis at the indicated time points. Already after 24 hours, morphological analysis of cells transfected with AATF-GFP displayed a much higher amount of detached and dead cells compared to cells transfected with the empty GFP vector (I, Fig. 1A), indicating that AATF can in this system promote apoptosis. Interestingly, a 2-fold increase in the amount of dead cells in cells over-expressing AATF is occurring already in the absence of an apoptotic stimulus and could for example indicate that high level of AATF could saturate regulatory factor(s)which would than be insufficient to regulate and prevent unwanted AATF activity.To quantitate these results, we performed FACS counting of the number of transfected cells stained for active caspase-3 and FACS counting of the number of transfected cells double stained for AnnexinV and Propidium Iodide (PI), both established markers for apoptosis (I, Figures.1B and 1C). Both quantitative analysis confirmed that AATF was able to sensitize cells to apoptosis by up to 3-fold when compared to cells transfected with the control vector. The higher amount of dying cells within the AATF-GFP population was increasing in a time-dependent manner and involved caspase-3 activation, as shown both by FACS counting of the number of cells labeled for active caspase-3 (I, Fig. 1B) and by the western blot analysis of the respective immuno-lysates blotted against cleaved and activated caspase-3 (I, Fig. 1D). In the future, it would be of interest to verify if AATF-mediated enhancement of apoptosis is caspase-dependent, for example by inhibiting caspases activation with a selective inhibitor, like z-VAD. This aspect was not addressed in the article and will not be further developed in this thesis.

1.3 AATF promotes c-Jun activation upon stress induction

Considering the well-known involvement of the AP-1 transcription factors in the UV response and as apoptosis mediators, we wanted to verify whether the marked increase in cell death upon AATF overexpression could correlate with an increase in the phosphorylation and activation of this family of transcription factors. To this end, we compared by western blotting the activation of c-Jun and ATF-2 using antibodies recognizing their phosphorylated and activated forms, ph-S73-c-Jun and ph-Thr-69/ 71-ATF2, in cells overexpressing AATF-GFP or GFP empty vector exposed to UV irradiation. Cells overexpressing AATF-GFP displayed a more pronounced phosphorylation and activation of c-Jun compared to the control vector but a similar activation potential of the other AP-1 family member, ATF-2 (I, Fig. 2A). Moreover, AATF-GFP overexpression was able to maintain c-Jun activated for up to 72 h, whereas in cells overexpressing GFP, c-Jun phosphorylation began to decline after 24 hours (I, Fig. 2C). These findings suggest a plausible link between AATF sensitization to UV-mediated apoptosis and c-Jun enhanced

activation, whose pro-apoptotic function is thought to be associated to its sustained and prolonged activation (Hibi, Lin, Smeal, Minden, & Karin, 1993) (Chen, Wang, Templeton, Davis, & Tan, 1996).

In parallel, we decreased the expression levels of AATF by siRNA transfection and found that the strength of c-Jun activation was proportional to the levels of AATF protein expression. We downregulated AATF in HEK293 cells using a pool of four different AATF siRNAs and verified the decrease in AATF expression levels both by western blotting and by semi-quantitative RT-PCR (I, Fig. 2 B, D and G). Cells were then exposed to 100 µJ UV radiation and harvested for analysis at the same time points analyzed for AATF-GFP overexpression experiment. Western blot analysis showed that the blockage of AATF expression completely abrogated activation of c-Jun both at an early stage of the UV-response and at later time points (I, Fig. 2B and D), indicating that JNK-mediated phosphorylation of c-Jun is dependent and proportional to AATF expression levels. We next analyzed the activity of a c-Jun construct which has the c-Jun DNA binding domain replaced by the DNA binding domain of the yeast transcription factor Gal4 (Gal4-c-Jun), coupled to a luciferase-based reporter assay. This construct allowed us to focus only on c-Jun transactivation capacity and to exclude an effect of AATF in regulating c-Jun affinity to its specific DNA *cis* regulatory elements. Results showed that increasing amounts of AATF-GFP were able to promote the transcriptional activity of the Gal4-c-Jun construct in a proportional manner but it had no relevant effect on the induction of the Gal4-dbd (DNA binding domain) construct or when AATF-GFP was replaced by the GFP empty vector (I, Fig. 2E). These results showed that AATF is promoting the activation of c-Jun transcriptional potential independently from c-Jun capacity to bind to its DNA responsive elements. Different regulatory mechanisms governing c-Jun transcriptional activity have been described in literature. The best known is the phosphorylation of c-Jun N-terminal region by the c-Jun N-terminal kinases (JNK) which interestingly seems to be necessary for activating the pro-apoptotic function of c-Jun (Ham et al., 1995; Le-Niculescu et al., 1999; Watson et al., 1998), for allowing the interaction with the RNA helicase RHII/ Gu during a stress response (Westermarck et al., 2002) and for promoting the dissociation of the HDAC3-containing repressor complex (Weiss et al., 2003). Of note, both AATF and the RNA helicase RHII/ Gu are localized in the nucleoli and both increase their nucleoplasmic localization upon a stress stimulus (I, Fig. 4D) (Westermarck et al., 2002), suggesting a plausible cooperative action for AATF and RHII/ Gu in the regulation of c-Jun transcriptional activity upon an apoptotic insult.

The identification of pro-apoptotic targeted genes upregulated by AATF in a c-Jun-dependent manner would be instrumental in understanding the physiological relevance of AATF stimulatory effect on c-Jun activity. The identity of the gene(s) necessary for c-Jun mediated apoptosis is not clear. However, the pro-apoptotic genes *Fas-L* and *TNF-α* can be induced by c-Jun and constitute therefore reasonable candidate apoptosis mediators (Fan & Chambers, 2001).

Hence, we sought to investigate whether AATF was able to modulate the induction of the well-known c-Jun target genes, *Fas-L* and *Tnfα*. To this end, we either overexpressed AATF-GFP or downregulated endogenous AATF in HEK293 cells and exposed cells respectively to 100 or 140 µJ UV irradiation. After 12 hours, cells were collected for analysis of *Fas-L* and *Tnfα* mRNA levels by semi-quantitative RT-PCR. Results showed that modulation of AATF expression level by overexpression or downregulation by AATF siRNA in UV-irradiated cells was directly proportional to the induction of *Fas-L* and *TNF-α* mRNA levels (I, Fig. 2F-G). Cells from the same experiment were harvested after 24 h for western blot analysis using

an antibody against Fas-L. In cells overexpressing AATF-GFP we observed a robust increase in Fas-L protein levels when compared to cells transfected with GFP empty vector (Figure 2).

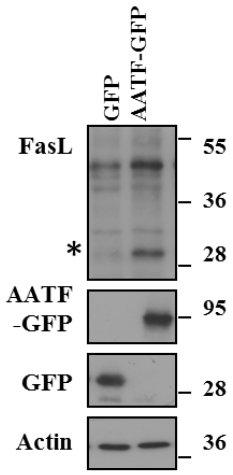

Figure 2. AATF regulates induction of c-Jun target gene Fas-L. HEK293 cells were transfected with AATF-GFP or GFP empty vector and exposed to 100 µJ UV irradiation. After 24 h cells were collected for western blot analysis against Fas-L. AATF-GFP and GFP blots show a similar transfection efficency and actin serves as a loading control. The star (*) indicates the cleaved Fas-L fragment.

The fact that depletion of AATF was able to prevent the induction of *Fas-L* and *Tnfα* (I, Fig. 2G and data not shown), suggests that AATF could function as a regulator of c-Jun transcriptional activity. To verify that AATF-mediated induction of *Fas-L* and *TNF-α* was due to an effect on c-Jun transcriptional activity and not on another transcription factor, we compared the induction of *TNF-α* in wt MEF and in c-Jun-deficient (c-Jun-KO) MEF cells overexpressing AATF-GFP or GFP empty vector (I, Fig. 2H-I). *TNF-α* mRNA levels were analyzed by semi-quantitative RT-PCR from cells harvested 24 hours after transfection and showed that *TNF-α* mRNA is induced upon AATF overexpression only in wt MEFs and not in c-Jun-KO MEF cells. Moreover, the reintroduction of c-Jun in c-Jun-KO MEFs restored the expression of *TNF-α* only when AATF was coexpressed with c-Jun (I, Fig. 2H). Together, these results provide further evidence that AATF promotes c-Jun-mediated induction of the pro-apoptotic genes *Fas-L* and *Tnfα* and is in line with our findings showing an increased cell death rate in cells overexpressing AATF (I, Fig.1). Finally, we examined whether AATF was able to promote the binding of c-Jun to the *Fas-L* and *Tnfα* gene promoter. To this end, we performed chromatin immunoprecipitation of endogenous c-Jun from UV treated HEK293 cells transfected with AATF-GFP or GFP. As a positive control for transcriptionally active chromatin region, we used antibodies against acetylated Histone 4 (Ac-H4) and as a negative control rabbit IgG (IgG) (Figure 3). ChIP assays revealed an increased association of c-Jun to the *FasL* and *Tnfα* gene promoter when AATF was overexpressed, suggesting that AATF may promote c-Jun recruitment to target gene promoters.

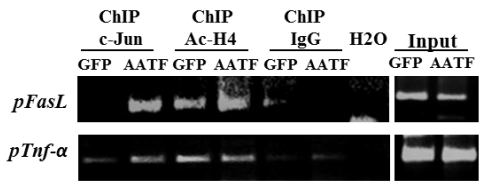

Figure 3. AATF promotes binding of c-Jun to *Fas-L* and *Tnf-α* promoter. HEK293 cells were transfected with GFP or AATF-GFP and exposed to 100 µJ UV irradiation. After 2 h samples were processed for ChIP analysis. Chromatin was immunoprecipitated with the indicated antobodies followed by PCR using primers for *Fas-L* or *Tn-fα* promoters, *pFasL* and *pTnf-α*, to examine the recruitment of c-Jun.

In summary, these findings identify AATF as a novel regulator of the proapoptotic functions of c-Jun. It would be of interest in the future to further investigate and pinpoint the pathway leading from AATF-c-Jun activation to apoptosis. For example by inhibiting Fas-L or TNF-α with a specific blocking antibody or by using Fas-L or TNF-α deficient cells, it could be possible to discriminate whether AATF-induced apoptosis is relying on Fas or on TNF-α mediated signaling. Also, considering the multiple processes activated by TNF-α and Fas-L, it would be of interest to investigate a possible involvement of AATF in regulating other processes promoted by Fas-L and TNFα, such as proliferation and differentiation of some cell types (reviewed in Aggarwal, 2003; Schuetze *et al*., 2008 and in the Review of literature section).

1.4. AATF promotes cell death in a c-Jun-dependent context

A clear link between AATF-mediated c-Jun activation and apoptosis came with the use of c-Jun-deficient (KO) MEF cells. As cells lacking c-Jun were impaired in AATF-mediated induction of *Fas-L* and *TNF-α* (I, Fig. 2 H-I), we investigated whether c-Jun deficient cells were also impaired in the AATF-mediated sensitization to UV-induced apoptosis. We reasoned that if AATF was promoting apoptosis by stimulating c-Jun-mediated induction of the pro-apoptotic genes *Fas-L* and *TNF-α,* than in a c-Jun deficient context, AATF would lose its capacity to enhance apoptosis. To test this hypothesis, we overexpressed AATF-GFP or GFP empty vector in wild-type or c-Jun-KO MEF cells and the following day we exposed cells to 15 µJ UV irradiation. Irradiated and untreated cells were collected after 48 hours for assessment of cell death. FACS analysis of the number of cells stained for AnnexinV and PI showed a marked increase of dead cells only when AATF-GFP was overexpressed in wild-type MEF cells and not in c-Jun-KO cells (I, Fig. 3). The number of dying cells was three times higher when AATF-GFP was expressed in wild-type cells than in c-Jun-deficient cells. These results provide evidence that the pro-apoptotic effect of AATF is dependent on the presence of c-Jun and further suggest that it could be mediated by the previously described increase in expression of pro-apoptotic-c-Jun target genes, such as *Fas-L* and *TNF-α* (I, Fig. 2 F-I).

1.5. Endogenous AATF interacts and co-localizes with c-Jun during the UV response

As AATF was able to promote apoptosis in a c-Jun-dependent manner, we sought to determine whether AATF was able to interact with c-Jun. To this end, we performed immunoprecipitation analysis of endogenous c-Jun or endogenous AATF from HEK293 cells UV-irradiated and harvested at different time points. Western blots against endogenous AATF or endogenous c-Jun respectively, showed an induced but transient interaction between c-Jun and AATF at an early phase of the UV response (I, Fig. 4 A and B). The interaction was more pronounced at an early stage, and decreased during the time course analyzed. Interestingly, the interaction seemed to precede the increase in phosphorylated c-Jun, indicating that AATF might determine the amount of c-Jun which can be

phosphorylated by JNKs, for example by promoting the release of c-Jun inhibitory complex or by ferrying key scaffolding or regulatory factors to the c-Jun complex.

In parallel, we investigated whether AATF change localization upon UV exposure. Confocal microscopy images of cells stained for endogenous AATF and nucleolin at different times points of the UV response, indicated that AATF is a nucleolar-localized protein in the absence of stress stimuli (I, Fig. 4D). After UV irradiation, the localization of AATF becomes increasingly nucleoplasmic and, in most cells, showed a complete loss of nucleolar localization after four hours of UV treatment (I, Fig. 4D). Interestingly, the increase in nucleoplasmic AATF upon UV-irradiation corresponded to an increase in colocalization with c-Jun that remained diffusely nucleoplasmic throughout the time course (I, Fig. 4E). Together, these results indicate that the asymmetric subcellular distribution of AATF and c-Jun might serve as a constraint to avoid unwanted or deregulated activation of c-Jun by AATF interaction. Similarly, several regulators such as MDM-2 and VHL (Mekhail et al., 2004; Sherr, 2006) are sequestrated in the nucleolus under normal growth conditions and are released upon stimulus to allow for interaction with their nuclear target proteins (Olson, Dundr, & Szebeni, 2000; Pederson & Tsai, 2009). The absence of a membranous border and the central localization within the nuclear space, render the shuttling of proteins from and to the nucleolus not only rapid, but also affordable in terms of ATP consumption (Stark & Taliansky, 2009). Interestingly, the interaction between endogenous AATF and endogenous c-Jun upon irradiation is transient, occurs at an early stage of the UV response and is lost at the latest time point analyzed (4 hours) (I, Fig. 4A and B). Instead, endogenous AATF retains a nucleoplasmic localization also 4 hours after UV irradiation, when the interaction with c-Jun is lost and even though both AATF and c-Jun are localized in the nucleus (I, Fig. 4D and E). These findings suggest the presence of additional regulatory mechanism(s) to adjust the amount and duration of AATF and c-Jun association in the nucleus, and thereby set a controlled c-Jun transcriptional activation and consequent gene expression profile.

1.6. Characterization of AATF functional domains

We next generated AATF deletion mutants to map the domains of AATF mediating the interaction with c-Jun. To this end, we generated two mutants harboring C-terminal truncations, one encoding exons I-X, AATF-GFP(1-454) and one encoding exons I-IV, AATF-GFP(1-198), and one mutant harboring a N-terminal truncation encoding exons V-XIII, AATF-GFP(199-526). In addition, we made use of an AATF isoform cloned from muscle cDNA, AATF-GFP(Δ125-246), which lacks exons IV-V (Figure 4). We transfected HEK293 cells with our AATF-GFP mutants and performed immunoprecipitation analyses of endogenous c-Jun to identify the domains of AATF required for binding to c-Jun. Blotting with an anti-GFP antibody the c-Jun immunoprecipitates from transfected cells , showed that AATF-GFP(1-454) and AATF-GFP(199-526) were able to associate with endogenous c-Jun, while AATF-GFP(1-198) failed to bind to c-Jun (I, Fig. 5A). These results indicate that AATF amino acids 199-454 are necessary to allow the interaction with c-Jun.

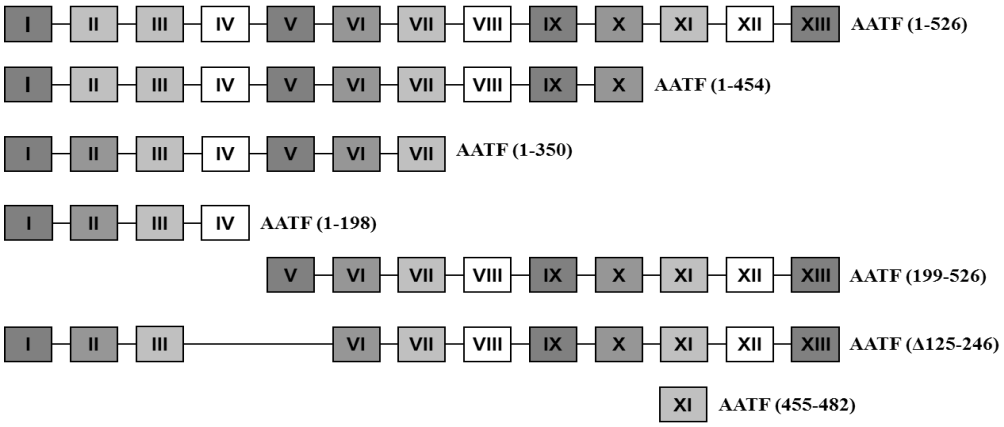

Figure 4. Schematic representation of the generated AATF deletion mutants. For each AATF mutant the encoding exons and the corresponding translated amino acids are shown

We next tested the capacity of the AATF deletion mutants to stimulate an AP-1-responsive luciferase reporter plasmid. While expression of wild-type AATF led to a robust increase in AP-1 activity, both our C-terminal truncation mutants, AATF-GFP(1-454) and AATF-GFP(1-198) were unable to stimulate AP-1 activity. In contrast, the N-terminal truncated mutant AATF-GFP(199-526) and also the AATF-GFP(Δ125-246) promoted c-Jun activity at levels comparable or higher than the full-length AATF-GFP(1-526) (I, Fig, 5B). Taken together, these results indicate that the C-terminal tail of AATF, encompassing amino acids 455-526, is necessary for stimulation of the AP-1 activity.

The identification of amino acids 455-526 of AATF as the region necessary to enhance c-Jun activity, prompted us to investigate whether the different capacity to stimulate AP-1 activity was directly proportional to the capacity to promote apoptosis. For this purpose, we overexpressed the AATF-GFP deletion mutants in MEF cells and determined the degree of apoptosis by FACS counting of cells double stained for PI and AnnexinV. In accordance with the previous AP-1 reporter results, cells transfected with AATF-GFP(199-526) and AATF-GFP(Δ125-246) deletion mutants, that stimulated AP-1 promoter activity, displayed a high number of dead AnnexinV-positive cells, whereas cells transfected with the C-terminal truncated deletion mutants AATF-GFP(1-454) and AATF-GFP(1-198), that did not stimulate AP-1 activity, were also defective in promoting apoptosis as shown by the lower number of AnnexinV-positive cells (I, Fig. 5C). However, the pro-apototic effect of the AATF mutants could also be mediated by other factors than c-Jun. To exclude this possibility, we decided to test the capacity of the AATF mutants to induce apoptosis in c-Jun-deficient MEF cells (c-Jun-KO MEFs), reasoning that if the AATF mutants were regulating the activity of some other apoptotic factors than c-Jun, we would detect a similar cell death rate in wild type and c-Jun-KO MEFs. To this end, we overexpressed AATF mutants in c-Jun-KO MEFs and, the following day, we harvested both floating and adherent cells for FACS analysis of dead cells. FACS counting of AnnexinV positive cells and western blot analysis for caspase-3 cleavage showed that in a c-Jun-deficient background, all the AATF mutants were unable to promote apoptosis, providing strong evidence for the interplay between AATF and c-Jun and for placing c-Jun as a downstream target of AATF (I, Fig. 5C and data not shown).

These results indicate that the ability of AATF to sensitize cells to apoptosis is dependent on its capacity to activate c-Jun transcriptional activity. Together, our results demonstrate that AATF amino acids 199-453 mediate binding to c-Jun while AATF amino acids 455-526 are necessary for stimulating the AP-1 activity and for promoting apoptosis in a c-Jun dependent context.

To further characterize the AATF deletion mutants we analyzed the subcellular localization of the mutants in HEK293 and MEF cells. We performed analysis of confocal microscopy images of cells transfected with AATF deletion mutants and stained for DAPI to visualize the nucleus, nucleolin to visualize the nucleoli or actin to visualize the cell morphology. Confocal images of cells transfected with AATF-GFP mutants showed that the N-terminal deletion mutant AATF-GFP(199-526) had a nucleolar localization similar to the nucleolar distribution of the full length AATF-GFP(1-526), whereas the C-terminal truncated isoform, AATF-GFP(1-454) showed a nucleoplasmic distribution and was excluded from the nucleoli (I, Suppl. Figure X). These results suggest that AATF C-terminal tail (AATF amino acids 455-526) is important to target AATF to the nucleoli. Interestingly, AATF exon XI, encoding for amino acids 455-482, contains a series of basic amino acids, such as the repetition of lysine residues which are typical for nucleolar targeting sequences (Birbach, Bailey, Ghosh, & Schmid, 2004; Reed et al., 2006).
To identify the region of AATF required for the nucleolar and nuclear targeting, we generated the following GFP-tagged constructs: AATF(1-350)-GFP encoding the first 7 exons of AATF and containing a putative nuclear targeting sequence (Passananti et al., 2007) and AATF(455-484)-GFP encoding AATF exons XI and containing a putative nucleolar targeting sequence (Figure 5). Confocal microscopy images of MEFs transfected with the deletion mutants revealed that while AATF(1-198)-GFP had an exclusively cytoplasmic distribution, AATF(1-350) showed an evident nuclear localization, indicating that the putative nuclear localization signal translated by exon VII (amino acid 290-310) is functional and sufficient to target AATF to the nucleus. Cells transfected with AATF(1-454)-GFP which lacks the potential nucleolar targeting sequence encoded by exon XI, display a nuclear but not nucleolar distribution. Instead, cells transfected with the construct AATF-GFP(455-482) showed that the fusion of AATF exon XI to GFP is sufficient to target GFP to the nucleoli and that AATF amino acids 455-482 contain a functional nuclear and nucleolar targeting sequence (Figure 5). It is tempting to speculate that different AATF isoforms could have distinct effects on c-Jun activity, providing a wide range of tissue-specific modes of c-Jun regulation. The characterization and identification of their tissue-specific conditions of expression will be of importance for cancer research.

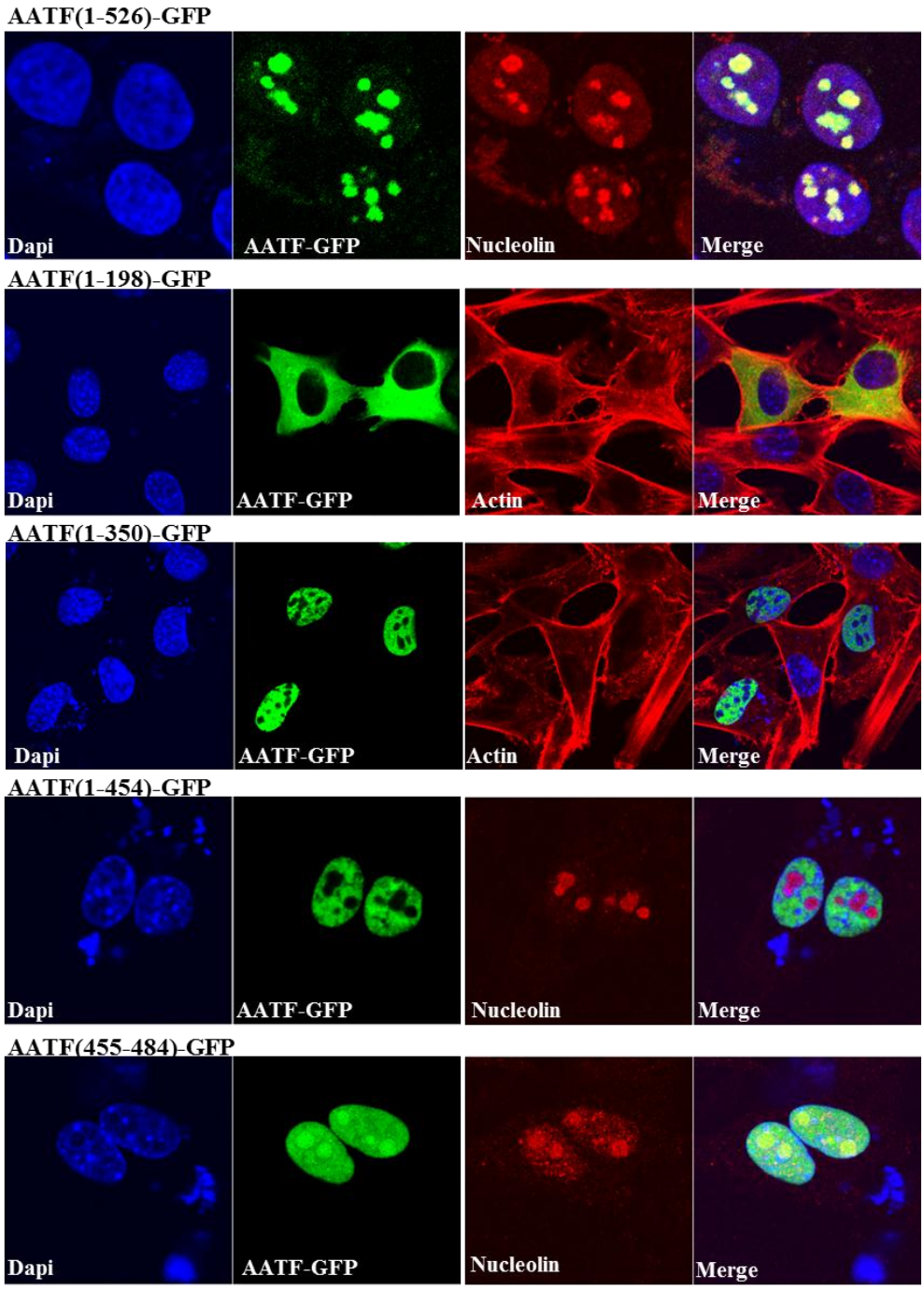

Figure 5. Identification of nuclear and nucleolar targeting sequence on AATF. MEFs were transfected with different AATF deletion mutants. The following day cells were fixed and stained with DAPI to visualize the nucleus, and actin to visualize the cell morphology or nucleolin to identify the nucleoli. AATF(1-526)-GFP which encodes the full length AATF isoform 1, shows a typical

nucleolar localization, AATF(1-198)-GFP has a exclusive cytoplasmic distribution while AATF(1-350)-GFP shows a nuclear localization, indicating the presence of a functional nuclear targeting signal. AATF(455-484)-GFP displays a nucleolar and nuclear localization identifying the region of AATF mediating nucleolar targeting.

Together, our results identify AATF as a novel regulator of the transcription factor c-Jun and reveal that AATF expression levels can determine the magnitude of phosphorylation and transcriptional activation of c-Jun upon several stress stimuli. AATF loses its nucleolar localization upon UV irradiation and transiently interacts with c-Jun in the nucleoplasm (Figure 6). Based on our analyses, amino acids 265-454 of AATF are required for the interaction with c-Jun while amino acids 455-482 of AATF are necessary to target AATF to the nucleoli. Moreover, amino acids 455-526 of AATF are also required to enhance c-Jun transcriptional activity and promote apoptosis in a c-Jun-dependent context, possibly through the induction of the proapoptotic genes *Fas-L* and *Tnfa*, whose products and activated signaling pathways could function as a likely mechanism of amplification of the apoptotic response.

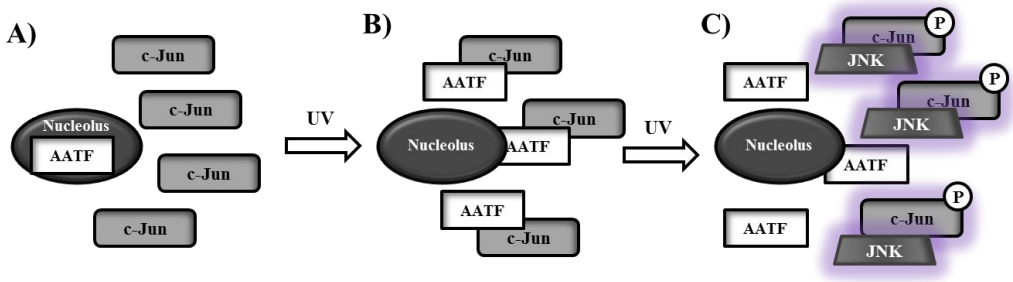

Figure 6. AATF regulates c-Jun activity. (A) Under physiological conditions AATF is retained in the nucleolus through a nucleolus targeting signal located in amino acids 455-484. **(B)** Upon UV irradiation AATF translocates into the nucleus where it interacts with c-Jun through AATF amino acids 199-454. **(C)** JNK can bind and phosphorylate c-Jun while AATF is released from c-Jun.

2. AATF activates c-Jun transcriptional activity in a JNK-independent manner

Activation of c-Jun has been classically attributed to JNK-mediated phosphorylation of c-Jun on residues Ser63 and Ser73 (Smeal et al., 1991; Smeal et al., 1992; Pulverer et al., 1991). The correlation between AATF expression levels and c-Jun phosphorylation status and transcriptional activation, prompted us to study whether AATF would stimulate c-Jun activity by enhancing the JNK kinases activities.

2.1 JNK activation is not affected by AATF expression levels

UV exposure promotes a rapid and strong activation of the JNK family, which results in the phosphorylation of c-Jun (Hibi et al., 1993). To investigate whether AATF was affecting the activation of the JNK pathway, we analyzed the activation of JNKs in UV-irradiated cells by western blotting, using a phospho-specific antibody recognizing phosphorylated and activated JNKs on residues Thr 183 / Tyr185. Blots showed that upon UV exposure overexpression of AATF enhanced the phosphorylation of c-Jun when compared to cells transfected with the GFP control vector, but did not significantly affect the phosphorylation status of JNKs (II, Fig.1), suggesting that AATF does not have a direct effect on JNK activation but rather acts downstream of it.

2.2. AATF regulates c-Jun activity in a JNK-independent manner

As AATF enhanced c-Jun phosphorylation without increasing the JNK activation, we determined if c-Jun phosphorylation could be mediated by another kinase or if AATF might promote c-Jun phosphorylation by another mechanism, for example by relieving the c-Jun associated inhibitory complex. To this end, we performed an UV-irradiation experiment with cells overexpressing AATF-GFP in presence of the JNK inhibitor SP-600125. We reasoned that if other kinases were involved in enhancing the phosphorylation status of the c-Jun N-terminal tail, the treatment with JNK inhibitor should not significantly influence c-Jun phosphorylation status upon UV exposure of AATF transfected cells. However, SP-600125 treatment was able to block AATF-mediated c-Jun phosphorylation, suggesting that the JNKs are the responsible kinases for AATF-promoted phosphorylation of c-Jun (II, Figure 2A).

Phosphorylation of c-Jun is associated with transcriptional activation and at least some functions of c-Jun seem to be dependent on its phosphorylation status, such as the capacity to induce apoptosis (Watson et al., 1998). We explored whether inhibition of c-Jun phosphorylation by SP-600125 treatment also inhibited AATF-mediated enhancement of c-Jun transcriptional activity. To this end, we made use of the previoulsy described c-Jun construct which has the c-Jun DNA binding domain replaced by the DNA binding domain of the yeast transcription factor Gal4 (Gal4-c-Jun), coupled to a luciferase-based reporter assay. This construct allowed us to study c-Jun transactivation capacity independently of the c-Jun DNA binding domain. We expressed the Gal4-c-Jun construct together with β-Gal, the luciferase reporter (pf-2-Luc), GFP or AATF-GFP, and analyzed the ratio of luciferase versus β-galactosidase activities. Surprisingly, the activity of the Gal4-c-Jun construct was unaffected by the inhibition of JNK activity when AATF-GFP was overexpressed, suggesting that JNK activity and phosphorylation is not required for AATF-mediated stimulation of the Gal4-c-Jun transcriptional activation (II, Fig. 2B). These results prompted us to investigate whether c-Jun phosphorylation was required for AATF to stimulate c-Jun transcriptional activity. To this end we made use of either a Gal4-c-Jun mutant construct defective in binding to JNK (Gal4-c-JunΔ) or of a Gal4-c-Jun mutant construct in which the JNK phospho target sites were replaced by alanine residues (Gal4-c-JunAA). Surprisingly, increasing

amounts of AATF-GFP were able to stimulate the transcriptional activities of both c-Jun phosphorylation-deficient constructs, Gal4-c-JunΔ and Gal4-c-JunAA, with a similar magnitude to the wild type Gal4-c-Jun construct (II, Fig.3C). These findings suggest that AATF stimulates c-Jun transcriptional activity independently of JNK-mediated phosphorylation of the c-Jun target sites Ser63/ 73.

2.3 HDAC1 promotes association of AATF to c-Jun

Phosphorylation-independent activation of c-Jun is poorly characterized. It is known that some functions of c-Jun do not require phosphorylation of residues 63/ 73. Indeed, unlike *c-jun*-deficient mice which die *in utero,* knock-in mice, expressing only a non-phosphorylable c-Jun S63/ 73A mutant (c-Jun-A63/ 73) are viable and develop normally (Hilberg, Aguzzi, Howells, & Wagner, 1993). Also MEFs derived from the c-Jun S63/ 73A mouse model grow poorly in culture and suggests that some functions of c-Jun do not require S63/ 73 phosphorylation (Wisdom, Johnson, & Moore, 1999). Weiss and coworkers provided results showing that JNK phosphorylation allows for the release of an inhibitory complex which normally keeps c-Jun inactive. They also showed that c-Jun inhibitory complex can be released by titrating the repressor by co-expression of a competitor such as c-Jun itself. In this case overexpressed c-Jun acts as a binding competitor for the inhibitor complex, thereby reliving the repressed state and, increasing c-Jun transcriptional activity in absence of JNK activity. While the regulation and composition of the c-Jun repressing complex remains to be determined, Weiss and coworkers have shown that HDAC proteins are important members of the inhibitory complex. Both HDAC1 and 3 can associate with c-Jun and associated HDAC3 keeps c-Jun transcriptional activity inhibited.

The above decribed findings prompted us to investigate whether AATF affected the association of the HDAC-containing inhibitory complex with c-Jun. For this purpose, we first performed immunoprecipitation analysis of cells overexpressing AATF-GFP and HDAC1-Flag or HDAC3-Flag and found that AATF associated with both HDAC1 and HDAC3 and endogenous c-Jun (II, Fig. 3A, unpublished data). Next, we sought to investigate whether AATF was able to affect the association of HDAC to c-Jun. Interestingly, overexpression of HDAC1 increased the association of AATF to endogenous c-Jun, suggesting that HDAC proteins might play an important role in determining the amount of AATF associated to c-Jun and thereby AATF-mediated regulation of c-Jun transcriptional activity (II, Fig. 3B). These results prompted us to explore whether HDAC could regulate AATF subcellular localization. Indeed, confocal microscopy analysis of HEK293 cells transfected with AATF-GFP and HDAC1 or 3 showed that both HDAC1 and 3 overexpression promote the shuttling of AATF or its stabilization in the nucleoplasm (II, Fig. 4). These results indicate that HDACs mediate the release of AATF from the nucleoli into the nucleus thereby increasing AATF interaction with c-Jun. Presumably, AATF nucleolar retention might be important to avoid unwanted c-Jun activation and could function as a tool to limit the duration and magnitude of c-Jun transcriptional activation. Future experiments will be directed towards understanding the precise nature of the regulation of c-Jun through AATF and HDAC-containing complexes. For example, it is not yet known whether the HDAC-mediated increase of AATF in the nucleoplasm, in the absence of any additional stress signals and/ or JNK phosphorylation, is sufficient to activate c-Jun transcriptional activity. Also, it will be of interest to explore whether the deacetylase activity of HDAC is necessary to enable the AATF release from the nucleolus. Indeed, acetylation can modify the structure of the protein and allows for selective and specific interactions with other factors that can in turn dictate for

example the retention in one compartment or the association to a specific cellular structure (reviewed in Glozak et al., 2005; Yang & Seto, 2007).

3. Protein kinase C ξ induces myoblast differentiation by targeting Cdk5/p25

Cell differentiation is a complex and multistep process which drives a proliferative single cell to exit the cell cycle and to engage into a tissue-specific and irreversible program of specialization which transforms a multipotent cell into a determined cell type. This process involves a myriad of interconnected pathways which need to be spatially and sequentially orchestrated to allow a complete rearrangement of the cell localization, morphology, recognition of fusion partner cell, and expression of tissue function-specific components (reviewed in Le Grand & Rudnicki, 2007). Our and other research groups have identified and described an essential role for the Cdk5/ p35 signaling complex in the differentiation of both neuronal and muscle progenitor cells. This last PhD project further develops and caractherizes the role of the Cdk5/ p35 complex in muscle differentiation and identifies in the atypical PKCξ, the upstream regulator.

3.1. Protein kinase Cξ activity is necessary for myoblast differentiation

The acquisition of a definitive protein expression repertoire and function, and hence of a cell type identity during differentiation, requires the inhibition and activation of several distinct pathways which allow for a complete change in cell morphology and phenotype.
Among the different pathways involved, we have focused on deciphering the roles of the atypical PKCξ, in regulating myoblast differentiation. Several studies have shown that PKCξ kinase activity is required for the proliferation, migration and differentiation of progenitor cells of different cellular lineages, but the downstream targets of PKCξ regulating these processes remain unclear (Calcerrada et al., 2002; White et al., 2002; Wooten et al., 1997). To assess the necessity of PKCξ activity also in the muscle differentiation model, we either downregulated PKCξ expression by siRNA (III, Fig. 1A) or blocked the kinase activity by treatment with a myristoylated pseudosubstrate peptide (PS) in myoblasts induced to differentiate (III, Fig. 1B). Confocal microscopy analysis of primary myoblasts showed a marked reduction in the number of myotubes and of cells stained for myosin heavy chain (MHC), a well-known marker for muscle differentiation, in cells transfected with siPKCξ or treated with the PKCξ inhibitor PS, when compared to the control conditions. Indeed, inhibition of PKCξ completely prevented the fusion of primary myoblasts and the formation of multinucleated myotubes typical of muscle differentiation. Western blot analysis of similarly performed experiments showed that the blockage of PKCξ activity hindered the expression of muscle differentiation markers, such as MHC, desmin and troponin (III, Fig. 2 and data not shown). Importantly, we performed similar differentiation experiments using CalphostinC, an inhibitor of nPKC and cPKC isoforms, and our results showed that inhibition of nPKC and cPKC had no effect on the differentiation process (III, Suppl. Fig. 1C), allowing us to focus on the involvement of the atypical isoform PKCξ.
These findings suggested an important role for PKCξ in regulating the fusion and differentiation of muscle progenitor cells.

3.2. Protein kinase Cξ activity increases during myoblast differentiation

As the depletion of PKCξ completely prevented the fusion of myoblasts and the expression of typical differentiation markers (III, Fig. 1 and 2), we investigated the levels of protein expression and of kinase activity of PKCξ during myoblast differentiation. To this end,

proliferating myoblasts were induced to differentiate by shifting to a differentiation medium and harvested at distinct time points. In primary myoblasts and C2C12 cells, western blot analysis showed that PKCξ protein levels remained constant during the analyzed 72 hours of differentiation (III, Fig 3A). In contrast, the kinase activity of PKCξ immunoprecipitated from differentiating myoblasts and analyzed using Histone1 as a substrate, showed that PKCξ kinase activity increases during the differentiation process and reaches a peak after 48 hours of induction (III, Fig. 3B). This finding indicates that PKCξ activity is upregulated during myoblast differentiation and that its kinase activity might therefore have an important role in regulating this process.

3.3 Protein kinase Cξ regulates Cdk5 activity during myoblast differentiation

Our and other previous studies have identified the Cdk5/ p35 signaling complex as an essential regulator of neuronal and muscle cell cycle arrest and differentiation (Cicero & Herrup, 2005) (Lazaro et al., 1997; Philpott, Porro, Kirschner, & Tsai, 1997; Sahlgren et al., 2003). We previously showed that Cdk5 activity is necessary for myoblast differentiation and for phosphorylation of the intermediate filament nestin on threonine 316. Cdk5-mediated phosphorylation enables the disassembly of nestin filaments which precedes and allows the cytoskeletal rearrangements necessary for cell migration and fusion into myotubes (Sahlgren et al., 2003). Interestingly, Cdk5 activity is induced during muscle differentiation and peaks at 48 hours with similar kinetics as PKCξ as previously described (Lazaro et al., 1997). Hence we decided to explore whether Cdk5 and PKCξ were interacting and regulating each other during myoblast differentiation and, if so, which of the two kinases was the upstream regulator. For this purpose, we analyzed kinase activities of PKCξ or Cdk5 in the presence of a Cdk5 inhibitor or of a PKCξ inhibitor respectively by immunoprecipitation of the kinases from myoblasts induced to differentiate. Kinase activity assays of immunoprecipitated Cdk5, using Histone 1 as a substrate, showed that inhibition of PKCξ activity prevented the induction of Cdk5 activity during myoblast differentiation (III, Fig. 4A). On the contrary, kinase activity of PKCξ immunoprecipitated from differentiating myoblasts showed an induction during differentiation also in the presence of the Cdk5 inhibitor Roscovitine. (III, Suppl. Fig. 2A). In fact, kinase assays using Histone 1 as substrate showed that immunoprecipitated PKCξ from differentiating myoblasts treated with the Cdk5 inhibitor Roscovitine had a similar phosphorylation activity when compared to PKCξ immunoprecipitated from non-treated myoblasts. Overall, these results strongly suggest that during myoblast differentiation PKCξ activity regulates the induction of Cdk5 and that, therefore, PKCξ is the upstream regulator of Cdk5 at least in the myoblast differentiation system. Accordingly, inhibition of PKCξ activity by treatment with the PKCξ inhibitor PS during myogenesis had an impact also on downstream Cdk5-regulated events. Indeed confocal microscopy images of differentiating myoblasts stained with nestin showed that inhibition of PKCξ impaired the reorganization of nestin filaments into long polymerized parallel fibers, which our previous work had shown to be mediated by Cdk5 phosphorylation (III, Fig. 4B). Western blot analysis of the corresponding lysates showed also that nestin expression and turnover was compromised and reduced by PKCξ inhibition or downregulation by siRNA (III, Fig. 4B and C). Overall, our results indicate that PKCξ functions as an upstream regulator of Cdk5 activity during muscle differentiation and show that PKCξ activity is necessary to allow cytoskeletal rearrangements leading muscle cell morphological changes that are regulated at least partially by Cdk5.

3.4. Cdk5 regulator, p35, interacts with and is phosphorylated by PKCξ

Cdk5 activity is completely dependent on the association with one of two activation subunits, p35 or p39 (Lew & Wang, 1995). p35 levels are regulated by proteasomal

degradation (Patrick et al., 1998; Saito et al., 1998), similarly to the regulation of cyclins in proliferating cells. However, the molecular mechanisms involved in the synthesis and degradation of p35 remain to be elucidated. The association of p35 to Cdk5 and the cleavage of p35 into the more active fragment p25are the key events which trigger Cdk5 activity. Therefore, we decided to explore whether PKCξ was targeting and regulating the Cdk5 activator p35 during myoblasts differentiation. First we sought to investigate whether p35 and PKCξ can interact with each other. To this end, we immunoprecipitated p35 or PKCξ from Cos7 overexpressing p35 and PKCξ and blotted against PKCξ or p35, respectively. In both cases, the blots showed that p35 and PKCξ are binding proteins (III, Fig. 5A). Next, we explored whether endogenous p35 and PKCξ are interacting in differentiating myoblasts. PKCξ was immunoprecipitated by p35 in differentiating C2C12 and importantly, this association was specific for differentiating myoblasts while in proliferating myoblasts the interaction was minimal (III, Fig. 5B). The fact that p35 and PKCξ associate during differentiation prompted us to verify whether p35 could be phosphorylated by PKCξ. To this end, we performed *in vitro* PKCξ kinase assays using as substrate recombinant p35 peptides corresponding to p35 amino acids 1-120 and p35 amino acids 208-307. Results showed that only p35 N-terminal domain p35(1-120), which contain the fragment removed upon cleavage, is phosphorylated by PKCξ, while p35(208-307) is not a target of PKCξ (III, Fig. 5 D). Next, mass spectrometric analysis of the in vitro phosphorylated p35 identified Ser33 as one of the main p35 target sites of PKCξ (III, Suppl. Fig. 4). The identification of this novel p35 phospho-site prompted us to generate a mutated p35 construct in which Ser33 was mutated to alanine in order to characterize the function of this phosphorylation site during differentiation.

3.5. PKCξ activity regulates calpain-mediated cleavage of p35 during myoblasts differentiation

As the activity of both Cdk5 and PKCξ increases with similar kinetics during myoblast differentiation and reaches peak levels after 48 h, we hypothesized that an increase in p35 cleavage could explain the described enhanced activity of Cdk5. We therefore examined the expression levels of p35 and p25 in differentiating myoblasts in the presence or absence of the PKCξ inhibitor PS. Western blot analysis of differentiating myoblasts showed that p35 is cleaved into the more active fragment p25 during differentiation but this cleavage is blocked when PKCξ is inhibited or downregulated by siRNA (III, Fig. 6A and C). These findings indicate that PKCξ is involved in promoting the cleavage and activation of p35 during myoblast differentiation. The proteolytic processing of p35 is mediated by calpains (Kusakawa et al., 2000), calcium-activated proteases whose activity has been reported to be involved in cell death and in muscle differentiation (Balcerzak et al., 1995; Liang et al., 2006). During myogenesis calpains cleave cytoskeletal proteins including vimentin, desmin, talin and fibronectin which regulate cell morphology, migration (Leloup, Daury, Mazères, Cottin, & Brustis, 2007) and fusion (Dourdin et al., 1997; Honda, Masui, Kanzawa, Tsuchiya, & Toyo-oka, 2008; Liang et al., 2006). Calpains do not use specific consensus target sequences for cleavage but they generally recognize motifs between conformational domains. In the case of p35, calpains recognize and cleave the linker connecting the N-terminal binding region and C-terminal p35 globular activation domain (Tarricone et al., 2001). In our study, we confirmed that the p25 fragment generated during myoblast differentiation was mediated by calpains as treatment of differentiating myoblasts with calpain inhibitor (Calpain Inhibitor III) was able to reduce p35 cleavage induced by differentiation (III, Fig. 6B). Also calpains are activated by autocleavage of their regulatory domain and the identification of a cleaved fragment of 75 kDa, corresponding to the catalytic domain, is used as a marker of calpain activation. Surprisingly, we found that inhibition of PKCξ activity by PS treatment or by downregulation of PKCξ expression by siRNA reduced the cleavage and activation of

both the ubiquitous calpain-1 and the muscle-specific calpain-3 (III, Fig. 7 A and B), suggesting that PKCξ activity regulates the activation of calpains during differentiation. These findings prompted us to investigate whether PKCξ is regulating the activity of calpains by direct interaction and phosphorylation. Immunoprecipitation analysis of endogenous PKCξ and calpain-3 showed that indeed PKCξ and calpain-3 are interacting proteins in myoblasts (III, Suppl. Fig. 7C). Moreover, kinase assays showed that recombinant PKCξ phosphorylates both immunoprecipitated calpain-1 (III, Fig. 7C) and calpain-3 (III, Suppl. Fig. 7B), indicating a promoting role for PKCξ in the regulation of calpain activation by interaction and phosphorylation. To substantiate these results showing a role for PKCξ in regulating calpain activities, we identified another substrate of calpains, RhoA (Kulkarni et al., 2002; Castellani et al., 2006), whose cleavage during differentiation was also blocked by PKCξ inhibition (III, Suppl. Fig. 7D). These results indicate that PKCξ functions as a regulator of calpain activation during myoblast differentiation. Considering that PKCα and δ, but not PKCξ, are regulated by calpains, it is tempting to speculate that calpain and PKC activities are propelled by a feed-forward mechanism (Hong et al., 1995; Liang et al., 2006). Therefore, PKCξ could function as an upstream regulator of calpains which in turn cleave and activate the PKCα and δ isoforms, further amplifying the signal and downstream activated pathways

3.6. PKCξ-mediated phosphorylation of p35 promotes calpain-mediated cleavage

Finally, we wanted to test whether the identified p35 phosphorylation site targeted by PKCξ has a role in regulating the cleavage of p35. For this purpose, we examined the susceptibility of the p35-S33A mutant to calpain-mediated cleavage by inducing calpain activity in transfected C2C12 myoblasts in the presence of the calcium ionophore A23187. Western blot analysis of transfected cells treated or not with the calcium ionophore showed a significant decrease in the cleavage of the transfected mutant p35-S33A when compared to the wild type p35 (III, Fig. 8A). Moreover, the comparison of the generation of the p25 fragment from cells overexpressing the wt p35 and cells overexpressing the mutant p35-S33A indicated that the phosphorylation of this site is important to allow the calpain-mediated cleavage of p35. Furthermore, in an *in vitro* cleavage assay the pre-incubation of the Cdk5/ p35 complex with recombinant PKCξ enhanced calpain-mediated cleavage of p35 regardless of Cdk5 activity, confirming that PKCξ-mediated phosphorylation of p35 plays a promoting role in the formation of the p25 fragment (III, Fig. 8B). Overall, these results suggest that upon differentiation PKCξ interacts and phosphorylates p35 on serine 33, thereby facilitating the calpain-mediated cleavage into the more active form p25.

In summary, we have identified PKCξ as a regulator of myoblast differentiation through targeting of the Cdk5/ p35 complex (Figure 7). Even though PKCξ deficient mice are viable and available for research studies, there are no reports about the involvement of PKCξ in muscle development. In fact, mouse models for PKCξ and Cdk5 have been characterized only for lymphoid organs (Leitges et al., 2001; P. Martin et al., 2002) and the CNS (Ohshima et al., 1996, 1999). The involvement of the aPKC subfamily in cellular differentiation has been explored in a neuronal cell model study showing that PKCι is a downstream target of PI3K and Src kinase and is necessary to allow nerve growth factor (NGF)-mediated induction of PC12 neuronal differentiation (Wooten, Seibenhener, Neidigh, & Vandenplas, 2000), and in a hematopoietic cell model study showing that PKCζ promotes erythroid differentiation of human monocytic U937 leukaemic cells (Mansat-De Mas et al., 2002). More recently, pharmacological or siRNA-based inhibition of PKCξ in myoblasts were shown to affect multiple phases of differentiation, including proliferation, migration and differentiation progression (Odemis, Boosmann, Dieterlen, & Engele, 2007). Moreover, PKCξ is essential in

regulating polarization and differentiation of mammary acinus ephitelial cells (Whyte et al., 2010) and to be critical in the self-renewal and commitment to tissue-specific differentiation of embryonic stem cells (Dutta et al., 2011). However, the likely compensatory role of the other aPKC, the ubiquitously expressed PKCι should be considered for in vivo studies of muscle development in the PKCξ deficient mice. Here we show that the cleavage and activation of the Cdk5 activator, p35 during myoblast differentiation is dependent on both PKCξ and calpain activities. We provide evidence for PKCξ functioning both by direct enhancement of calpain activities and by phosphorylating p35 into a calpain cleavage-permissive form. The prominent role played by PKCξ in myoblast differentiation, suggests an involvement of this kinase at multiple steps of this complex process. Moreover, the importance of both Cdk5 and PKCξ in regulating neuronal differentiation and homeostasis make the discovery of the signaling complex PKCξ/ Cdk5/ p35 of interest for further studies and characterization in neurodegenerative diseases.

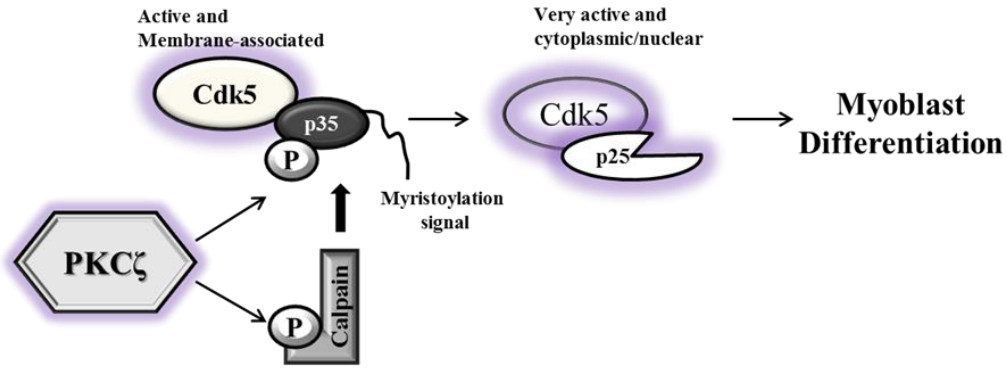

Figure 7. Schematic model depicting the role of PKCζ in myoblast differentiation. PKCζ phosphorylates and activates calpains on one side and phosphorylates p 35 on Ser33 which allows for calpain-mediated cleavage of p35 on the other side. p35 cleavage is responsible for the increase in Cdk5 activity and relocalization of the complex Cdk5/ p25 into the nucleus.

CONCLUDING REMARKS

The final outcome of the activation of a stress sensor is highly dependent on the cellular protein composition, and on the type and intensity of the stress. Stress signals are transmitted through a limited number of enzymatic stress pathways which have been optimized during evolution to allow a more diversified, fine-tuned and efficient response. The identification of a key player within a stress pathway and the mapping of its crosstalk potential with the neighbouring pathways is of obvious importance.

In this thesis, I identified in AATF a novel regulator of the AP-1 transcription factor c-Jun. The JNK-c-Jun pathway is a well-known mediator of cell adaptation to many environmental stresses and oncogenic transformation which are found regulating stress responses and differentiation in all branches of eukaryotes. For decades, JNK-mediated phosphorylation has been a dogma to explain c-Jun transcriptional activation. Yet phosphorylation-independent functions of c-Jun have been reported, but these have remained poorly understood. Here I identify in AATF a JNK-independent activator of c-Jun transcriptional activity, and show that AATF is required for c-Jun-mediated apoptosis and for the induction of the pro-apoptotic c-Jun target genes *FasL* and *Tnfα*. Importantly I show that AATF is a nucleolar protein which shuttles into the nucleus upon UV irradiation or HDAC overexpression. Asymmetric subcellular distribution of proteins is an excellent tool to increase the variability in composition and consequent function of multiprotein signaling complexes and evolved in parallel to the increase in cellular compartmentalization. The nucleolus is an ideal nuclear structure to store stress regulators as these can be rapidly released to transcription factor targets mediating a stress response, while adapting ribosomal production and cell growth to the sensed stress and to cellular energy supplies levels. Indeed yeast homologous AATF is a nucleolar protein involved both in stress response and ribosomal production. Highly complex and diverse stress response pathways have developed by increasingly complex multicellular organisms during evolution. Stress response pathways have optimized and diversified the mechanisms to sense environmental conditions and nutrient availability and respond with a rapid but controlled nucleolar release of key regulators to nuclear transcription factors, such as the here described c-Jun. The release of AATF into the nucleus could therefore be proportional to the stress levels and determine the magnitude of c-Jun-mediated biological responses.

Subcellular distribution of key signaling regulators is a critical aspect also in the second major topic of this thesis covering the induction of the differentiation process. All the studies of this PhD describe signaling regulators whose spatial distribution is regulated by specific stimuli and associated to a distinct protein function. Indeed, in the last article, I show that, upon induction of differentiation the signalling complex Cdk5/ p35 moves from the cytoplasm to the nucleus where it targets an unidentified differentiation key regulator. The differentiation process involves a massive redistribution of regulators and kinases which enable the resetting of the expression profile to the new cellular phenotype and is mediated by an intense and targeted protein destruction and synthesis to allow the shaping of the induced morphological and functional phenotype. Among the myriad proteins involved, we focused in identifying the upstream regulator of the Cdk5/ p35 complex. We show that the atypical PKCξ phosphorylation of p35 is necessary to allow calpain-mediated cleavage of p35 and consequent increase in Cdk5 activity during differentiation. Upon cleavage, the Cdk5/ p35 complex shuttles into the nucleus where Cdk5 plays a still unknown role during differentiation. Cdk5/ p35 shuttling to the nucleus has been described during an apoptosis

insult and Cdk5 has been shown to phosphorylate and inhibit the pro-survival activity of MEF2. Interestingly MEF2 activity is essential also to regulate muscle differentiation, suggesting one possible and probable candidate target of Cdk5 in the nucleus during differentiation. Moreover, we also showed that PKCξ phosphorylates and thereby regulates calpains activities during myoblast differentiation, suggesting that PKCξ may both select calpain cleavable targets by phosphorylating them and boost calpain activity. Indeed, the fact that PKCξ phosphorylates both calpains and their substrates suggest a plausible mechanism of regulation and selection of the substrates targeted by this protease upon distinct stimuli.

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