ANALYSIS OF THE ROLE OF EXTRACELLULAR SIGNAL REGULATED KINASE 5 (ERK5) IN THE DIFFERENTIATION OF MUSCLE CELLS

Dissertation

For the Completion of Doctorate Degree in Natural Sciences at the Bayerische Julius-Maximilians-Universität Würzburg

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I hereby declare that the submitted dissertation was completed by myself and no other. I have not used any sources or materials other than those enclosed.

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The hereby submitted thesis was completed from March 1999 until June 2001 at the Institut für Medizinische Strahlenkunde und Zellforschung, Bayerische Julius-Maximilians Universität, Würzburg under the supervision of **PD Dr. Stephan Ludwig** (Faculty of Medicine) and **Professor Dr. Werner Göbel** (Faculty of Biology).

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I. SUMMARY

The MEK5/ ERK5 kinase module is a relatively new discovered mitogen-activated protein kinase (MAPK) signalling pathway with a poorly defined physiological function. Since ERK5 and its upstream activator MEK5 are abundant in skeletal muscle a function of the cascade during muscle differentiation was examined.

ERK5 becomes activated upon induction of differentiation in mouse myoblasts. The selective activation of the pathway results in promoter activation of differentiation-specific genes, such as the cdk-inhibitor p21 gene, the myosin light chain (MLC1A) gene, or an E-box containing promoter element, where myogenic basic-helix-loop-helix proteins such as MyoD or myogenin bind. Moreover, myogenic differentiation is completely blocked, when ERK5 expression is inhibited by antisense RNA. The effect can be detected also on the expression level of myogenic determination and differentiation markers such as p21, MyoD and myogenin. Another new finding is that stable expression of ERK5 in C_2C_{12} leads to differentiation like phenotype and to increased p21 expression levels under growth conditions.

These results provide first evidence that the MEK5/ERK5 MAP kinase cascade is critical for early steps of muscle cell differentiation.

I. ZUSAMMENFASSUNG

MEK5/ ERK5 ist ein erst kürzlich entdeckter MAPK- Signalweg, dessen physiologische Funktion noch wenig verstanden ist. Da ERK5 und der in der Kaskade oberhalb liegende Aktivator MEK5 in Skelettmuskeln hoch expremiert werden, wurde eine Funktion der Kaskade während des Muskel-Differenzierung untersucht.

ERK5 wird nach einer Induktion der Differenzierung in Maus-Myoblasten aktiviert. Die gezielte Aktivierung dieses Signalwegs führt zur Induzierung von Promotoren differenzierungs-spezifischer Gene, wie z.B. des cdk-Inhibitors p21, der MLC1A, oder eines Promotors, der E-Boxen enthält, woran myogene Basische-Helix- loop- Helix Proteine, wie MyoD oder Myogenin binden können.

Darüber hinaus ist die Muskeldifferenzierung völlig blockiert, wenn die Expression von ERK5 mittels antisense-RNA inhibiert wird. Diesen Effekt kann man auch an hand der Menge von exprimierten muskelspezifischen Differenzierungsproteinen, wie p21, MyoD und Myogenin nachweisen.

Eine weitere neue Entdeckung ist, daß stabile Expression von ERK5 in C_2C_{12} Zellen zu einem differenzierungsähnlichen Phänotyp und gesteigerter p21 Expression unter Wachstum-bedingungen führt.

Diese Ergebnisse geben erste Anhaltspunkte, daß der MEK5/ ERK5 MAP Kinase Signalweg entscheidend für frühe Stadien der Muskeldifferenzierung ist.

II. Introduction

II-1. MAPK signalling pathways

Cells respond to changes in the surrounding environment by switching on/ off intracellular programs. Such a response needs to be quick, specific and spatially and temporally co-ordinated with the signals received from other cells or tissues, in order to induce an appropriate response to the challenge. To this end intracellular signalling pathways have evolved including the mitogen-activated protein kinase (MAPK) signalling cascades. Activation of MAPK signalling cascades represents an early mechanism by which cells transduce intracellular signals in response to various extracellular stimuli. Over a dozen MAPKs organised in at least four different families have been identified in mammals. They are activated via phosphorylation catalysed by distinct upstream kinases on both tyrosine and threonine residues within their catalytic domains. These processes are vital to the cells, therefore a high degree of homology exists between divergent species.

The mitogen-activated protein kinase (MAPK) pathways transduce various extracellular stimuli to mediate distinct intracellular responses. The core components of such a MAPK module is a set of three sequential acting kinases, which are evolutionarily conserved from plants to animals. In mammalian cells, three distinguishable MAPK modules have been well described now known as the ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase), and the p38/MAPK pathways. These pathways regulate a variety of responses including cell growth, differentiation, adaptation to the environment, as well as apoptosis [1-3]. The MAPKs also control numerous regulatory processes during development and homeostasis (reviewed in [4-6]).

The mammalian ERK module, also known as the *classical* mitogen cascade, consists of serine/ threonine kinase Raf, the dual-specificity kinase MEK, and the classical MAPK ERK. For both

MEK and ERK2, there exist two isoforms while three isoforms, A-, B- and C-Raf are known for the Raf kinase. In their protein sequence, ERK's contain a Thr-Glu-Tyr (TEY) motif and become strongly activated, through phosphorylation of the Thr and Tyr residues, after treatment with mitogenic stimuli such as growth factors.

Two other MAPK modules leading to the activation JNK and the p38 MAPK that possess a Thr-Pro-Tyr (TPY) motif or a Tyr-Gly-Tyr (TGY), respectively, respond to a variety of cellular stresses and proinflammatory cytokines [2, 7].

It has been shown that at least five kinase modules exist in yeast. The complexity of the mammalian genome would indicate that there maybe more than 50 MAP kinases in mammals. Searches that have been made to identify novel MAP kinase cascades using different molecular biology techniques revealed several "orphan" pathways. These include ERK3 kinase/ERK3, and ERK4.

Quite recently a novel-signalling pathway termed as MEK5/ ERK5, has been identified.

II-1.1. The Raf/MEK/ERK module

The Raf/MEK/ERK signalling cascade becomes strongly activated by mitogenic stimuli such as growth factors. This signalling pathway transduces signals leading to growth or differentiation in cells.

Binding of a growth factor e.g. epidermal growth factor (EGF) to the corresponding receptor tyrosine kinase (RTK) leads to its oligomerisation, and subsequent autophosphorylation. Phosphorylated tyrosines on the intracellular part of the receptor, serve as docking sites for SH2-domain (*Src homology*) of adapter protein Grb2, which itself binds via its SH3-domain to the proline rich motif of the GDP-GTP exchange factor Sos (*son of sevenless*). Sos interacts with Ras and activates the exchange of GDP to GTP [8-11].

GTP loaded Ras leads to the recruitment to the cell membrane of the cytosolic serine/threonine kinase Raf, a membrane shuttle kinase-identified as an oncogenic product of the murine sarcomavirus 3611 [12-16]. These translocation results in the activation of the membrane associated Raf. Ras-Raf interaction alone however, is not sufficient to activate Raf completely [17-19].

Raf regulation is complex involving protein-protein interactions, phosphorylation of tyrosine, threonine and serine residues and cellular relocalisation (for review see [20]). Activation mediated by multiple kinases like Src, PKC (protein-kinase C) and PAK (p21 (Rac/Cdc42)-activated protein kinase) leads to phosphorylation and positive regulation of Raf [21-25], whereas other kinases like PKA (protein kinase A) downregulate Raf activity [26].

The first characterised substrate of Raf isoenzymes was the dual specificity kinase MEK [27]. Once activated, MEK transduces the signals through phosphorylation on T-E-Y- motifs in the MAP-kinases ERK1 (p44) and ERK2 (p42) [28, 29]. The activation of MEK1 and ERK1 through Raf can be enhanced via interaction with the scaffold protein MP1 (*MEK partner 1*) [30].

ERK phosphorylation increases its catalytic activity, mediates oligomerisation and alleviates the shuttling of the kinase to the nucleus. ERKs are nuclear shuttle kinases and have several described substrates in contrast to its upstream activators Raf and MEK. Their target proteins are e.g. serine/threonine kinases like 3pK, ribosomal S6 kinase ($RSK1^{p90}$) also known as MAPKAP-Kinase 1; RNA-polymerase II, phospholipase A2 and several transcription factors like, Elk-1 and c-Jun [17, 31-34].

ERKs are essential elements of mitogenic signalling. Prolonged activation and nuclear retention of ERKs is required for transcription of the *cyclin D1* gene [35], suggesting a mechanism of

ERK-mediated enhancement of cell cycle entry. ERKs are also associated with cytoskeleton [36], playing a putative role in cytoskeleton reorganisation.

Expression of constitutively active components of the pathway cause cell transformation [1]. ERKs were active in renal carcinomas and their activity directly correlated with tumour grade [37]. MEK1 is also overexpressed in many tumours [37].

It has to be noticed that decision to proliferate or differentiate is taken upon the length of time that MAPK pathway is activated [38]. This mechanism has been proposed for regulating proliferation versus differentiation decisions depending on the cell type [38, 39]. In PC12 cells prolonged ERK activity leads to differentiation [40].

But this is not a general observation. In U937 cells phorbol ester promotes sustained ERK activation but not cell differentiation [41]. In keratinocytes, Ca^{2+} induces ERK transiently and this activation correlates with differentiation, while a more sustained EGF induced ERK activation is associated with proliferation [42].

II-1.2. The Stress-activated JNK- and p38 MAP Kinase Cascades

II-1.2.1. JNK- MAP Kinase Cascade

Pro-inflammatory cytokines like TNFα (tumour-necrosis-factor α) and IL-1 (interleukin-1) or stress stimuli like UV light, osmotic and heat shock, or chemical reagents like anisomycin and arsenite [7, 43] are strong activators of JNK - and p38 MAPK cascades.

Activation mediated by those stimuli leads to phosphorylation of numerous protein kinases, which function as MKKKs (MAP kinase kinase kinase). Those MKKKs such as: MEKK1, MEKK2 and MEKK3 (reviewed in [2]); SPRK/MLK3 (*SH3-domain-containing proline-rich kinase/mixed lineage kinase 3*) [44]; or protein kinase Tpl-2 (*tumour progression locus 2*) and its

human homologue Cot (*cancer osaka thyroid*) [45] were implicated in the activation of the JNK pathway. These MKKKs phosphorylate and activate preferentially two MKKs (MAP kinase kinase) MKK4 and MKK7 (*MAP kinase kinase*), which both reveal homology to MEK [46]. They in turn activate JNK by dual phosphorylation on Thr and Tyr.

A well known substrate for JNK is the transcription factor c-Jun. Phosphorylation by the kinase at the N-terminal serines S63 and S73 leads to its activation [47]. Transcription factors like ATF-2 and GABP become also activated through JNK like the kinase 3pK [33, 48, 49].

Scaffold proteins, play also an important role in mediating the spatial and temporal specificity of the stress cascade activation. Association of JNK with MKK7 and MLK3 is mediated by the scaffold protein JIP-1 (*JNK interaction partner* 1) [30, 50].

II-1.2.2. p38 - MAP Kinase cascade

Lipopolysaccharides, arsenite, heat- and osmolarity shock lead preferentially to activation of p38 MAPK [51-54]. The phosphorylation motif of this MAPK consists of a T-E-Y-sequence, which becomes activated through MKK3 and MKK6. Although MKK4 influences p38 activity, the decisive role in its activation plays MKK6 as shown *in vivo* [55]. p38 in turn, phosphorylates the MAPKAP kinases 2 and 3 [33, 56] as well as the MEF2 (myocyte enhancer factor) transcription factors [57]. The transcription factor ATF2 is also substrate for p38 [43].

II-1.2.3. Physiological roles of JNK and p38 MAPK cascades

JNK and p38 signalling pathways are implicated in several physiological processes spanning from cell differentiation [58, 59], IL-production [60, 61], to induction of apoptosis [62-64].

p38 kinase was shown in several studies to regulate the activity and thus to control differentiation processes such as myogenesis [57, 65-68]; or process of neuronal differentiation

[69]. p38 was also shown to take part in cardiovascular development [70, 71], suggesting a crucial role of phosphorylation as a process for regulating the MEF2 transcriptional activity.

New studies show that stress- and mitogen-induced kinase cascades are linked together at different points. Often the level of convergence of cascades occurs on the level of transcription factors like ATF-2 [72]. Some kinases like Tpl-2 [73] or MLK3 [74] are activating more than one downstream kinase. MAPKAP-kinases were also shown to receive signals from different MAP-kinases; this includes 3pK, MNK1/2 (*MAPK-interacting kinases 1/2*) and MSK1 (*mitogen- and stress-activated protein kinase-1*), which were activated trough ERK and p38 phosphorylation [33, 75-78].

II-1.3. MEK5/ERK5 signalling pathway

The MEK5/ ERK5 module was identified most recently as a novel member of the family MAPK signalling cascades.

II.1.3.1. MEK5 - identification, structure and function

Screening of a cDNA library from adult rat brain led to identification of a novel MEK named MEK5 [79, 80]. MEK5 exists in two alternatively spliced isoforms. The α-isoform encodes a 50kD protein, which shows a restricted expression pattern. The β-isoform has a molecular weight of 40kD and is ubiquitously distributed and primarily cytosolic [80] (Figure 1).

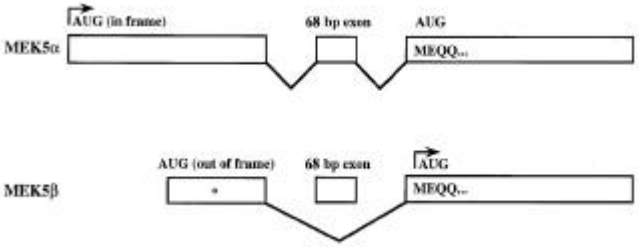

Fig. 1 Schematic representations of MEK5-a and MEK5-b. Top schematic, the inclusion of the 68-bp exon in the 5` untranslated sequence of MEK5 results in an upstream change in frame usage. This adds 89 amino acids to the predicted sequence of MEK5, yielding a protein of 448 amino acids. Bottom schematic, in MEK5, without the 68-bp exon, the first methionine downstream of the exon splice site is used for initiation yielding a protein of 359 amino acids.

Sequences located at the N-terminus of MEK5 suggest coupling of GTPase signalling molecules to the MEK5 protein kinase cascade (Figure 2), [79].

```
61   EYEDEDGDRITVRSDEE   77    MEK5
786  KYVDEDGDFITITSDED   802   scd1
696  KYQDEDGDFVVLGSDED   715   CDC24
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Fig. 2 Sequence alignment of MEK5 with the scd1 gene and CDC24 gene encoded proteins.

Genetic and biochemical studies have demonstrated that the CDC24 encoded protein has GDP release activity. When the CDC24 encoded protein binds to the CDC42 GTPase, it enhances the GTP/GDP exchange [81]. In a similar fashion, the scd1-encoded protein enhances the nucleotide exchange of the corresponding *S. pombe* encoded protein (cdc42sp). The binding domain of scd1 that recognises cdc42sp is located C-terminal to amino acid 671 of scd1 [82]. It is this region of scd1 that shows sequence identity to MEK5 as well as CDC24. Although the function of this sequence is unknown, it is possible that the N terminus of MEK5 that interacts with the mammalian equivalent of CDC42. The mammalian equivalent of yeast CDC42 is Rac [83]. The amino acid sequences in question may provide a mechanism for coupling GTPases (i.e. CDC42 and Rac) to downstream protein kinase signalling cascades.

The mouse MEK5 displays 99 and 93% identity with rat and human sequences respectively [79, 80]. The activating phosphorylation sites Ser-311 and Thr 315 are conserved in the species **(**Figure 3**).**

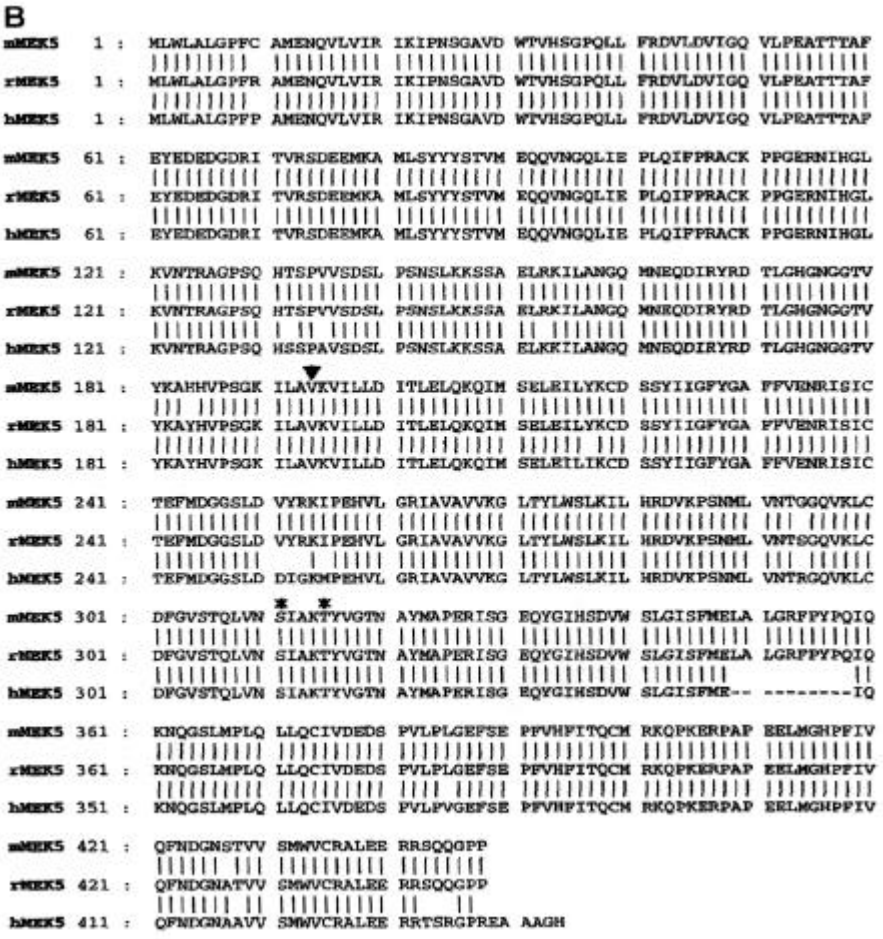

Fig. 3 Primary structure of mouse and human MEK5. Sequence alignments of mouse MEK5 with human MEK5. The identical amino acid residues were linked with *vertical lines*. Activating phosphorylation sites and ATP-binding sites are denoted by *asterisks* and *arrowheads* respectively.

The α isoform of MEK5 has a short stretch of amino acids termed AID motif (for atypical PKC-interaction domain) spanning from amino acids 61 to 78 on the MEK5 molecule (Figure 4).

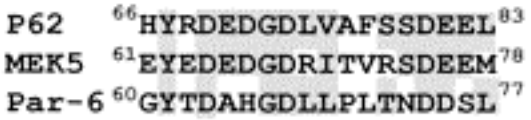

Fig. 4 The AID sequences of MEK5 and p62 aligned with the corresponding putative region of Par-6. Identical and closely related residues are shown in grey.

Via this domain MEK5 binds to PKCζand PKCλ/ι [84]. This interaction is EGF inducible and sufficient to activate MEK5. The AID domain has been found also in p62 which provides a

scaffold link between PKC and the tumour necrosis factor α (TNF-α) and interleukin-1 (IL-1) receptor signalling complexes mediating response to activation by those cytokines [85].

MEK5 has been shown to interact with MEKK3 [86], and its relative MEKK2 [87]. Although close homologues MEKK2 and MEKK3 activate the pathway in a specific manner depending on the cell system and stimuli [87].

II-1.3.2. ERK5 - identification, structure, and function

Studies performed using the yeast two-hybrid system helped to identify a novel MEK5-binding protein, termed ERK5, which specifically interacts with the dominant negative MEK5 mutants S311A/ T315A and K195M [79].

ERK5 is approximately twice the size of all known MAPKs with a length of 815 amino acids.

The mouse ERK5 shows 92% identity with human ERK5. The kinase domain, which is located at the N-terminus is highly conserved including the dual phosphorylation site (Thr 219 and Tyr 221) in the TEY sequence. The N-terminal domain from amino acids 1-77 is important for cytoplasmic targeting; a domain within amino acids 78-139 is required for association with the upstream kinase MEK5; and another region amino acids 140-406 is necessary for oligomerisation [88]. The C-terminus contains a putative NLS (nuclear localisation signal) suggesting a role in nuclear translocation of ERK5. A proline-rich motif is located in this region, which proposes possible targeting to the cytoskeleton **(**Figure 5**)**. Certain Pro-rich regions are also known to bind to SH3 (Src homology domain 3) domains. They are found in many proteins involved in tyrosine kinase signalling and cytoskeletal organisation (for review see [89]).

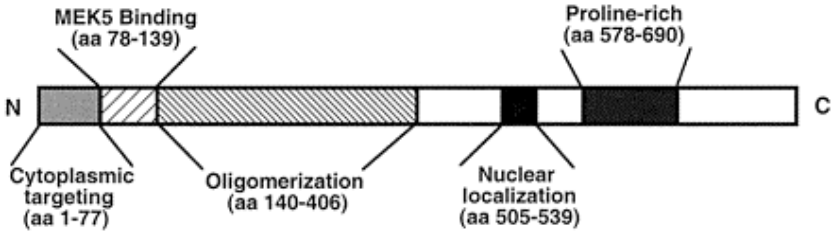

Fig. 5 Schematic diagram showing ERK5 organisation of functional domains.

Three differentially spliced isoforms of mouse ERK5 have been identified recently (Figure 6): mERK5a, mERK5b, and mERK5c. mERK5b and mERK5c function as dominant negative kinases blocking the activity of mERK5a [88]. Differential splicing of the mRNA or expression of the dominant-negative isoforms suggests a regulatory function.

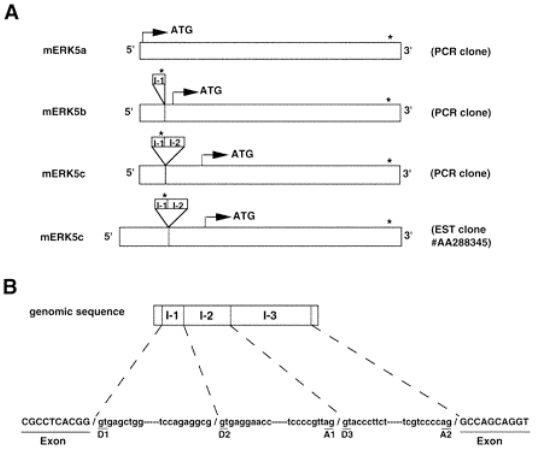

Fig. 6 Schematic representations of mouse ERK5 splice variants and genomic structure at the splicing junction. A, schematic diagram of mouse ERK5 cDNAs: I-1, I-2, and I-3 refer to the insertions of DNA sequence. The asterisk indicates the stop codon. B, schematic diagram of partial genomic DNA flanking the splicing region of mouse ERK5 gene. The splicing donor gt sites (D1, D2, and D3) and splicing acceptor ag sites (A1 and A2) are underlined. Exon and intron regions are represented by capital and lowercase letters, respectively.

ERK5 unlike ERK1/2 exists as oligomer in unstimulated cells [88]. In contrast to ERK5, ERK1/2 oligomerise upon phosphorylation. Whereas ERK5 overexpression in both stimulated and unstimulated cells leads to oligomerisation suggesting that oligomerisation does not depend on the phosphorylation status of the kinase.

Whilst possessing a TEY motif, and thus resembling the classical ERKs, ERK5 is strongly activated by osmotic, oxidative or fluid shear stress [90, 91]. Later studies revealed that growth factors like EGF [86, 92, 93] or NGF and serum [92] are also activators of ERK5.

Other studies place MEK5/ERK5 under the control of G protein-coupled receptors (GPCR) mediated by $G\alpha_q$ and $G\alpha_{12/13}$ subunits of heterotrimeric G proteins [94].

Novel studies revealed a putative role of MEK5/ERK5 in TNF-α promoter activation upon crosslinking of high affinity IgE receptor (FcεRI) with its corresponding ligand [95].

Activation mediated by diverse stimuli couples the MEK5/ERK5 signalling pathway to transcription factors enhancing their activity.

ERK5 has been shown to up-regulate the transcription from several immediate early gene promoters, e.g. the *c-jun* promoter [93, 95, 96], or the *c-fos* SRE (serum responsive element) via Sap1 [92]. Further ERK5 was implicated in c-myc phosphorylation [97]. Pearson et al. suggested a co-operation between MEK5/ERK5 and ERK2 to regulate NF-κB activity [98].

The best-characterised targets of ERK5 so far are the members of the MEF2 (myocyte enhancer factor) family. ERK5 interacted in a yeast two hybrid screen with MEF2 proteins and it phosphorylated in vitro MEF2C and MEF2D [99]. Further studies [100] revealed that, in addition to MEF2C, ERK5 phosphorylates and activates MEF2A and MEF2D but not MEF2B. The sites phosphorylated by activated ERK5 were mapped to Ser-355, Thr-312, and Thr-319 of MEF2A and Ser-179 of MEF2D both in vitro and in vivo. Phosphorylation of these sites in MEF2A and MEF2D are necessary for the induction of MEF2A and 2D transcriptional activity.

Fig. 7 Sequence comparison of members of the MEF2 group of transcription factors in the regions phosphorylated by ERK5. Gaps introduced into the sequence to optimise alignment are indicated by a hyphen. The numbers indicate the starting and ending amino acid residues of each MEF2 protein shown. Asterisks indicate amino acid residues phosphorylated by ERK5.

Interestingly, the MEF2 sites phosphorylated by ERK5 are not conserved with respect to each other (Figure 7). This suggests that the general structural similarity among MEF2 proteins may only contribute to their initial recognition/ interaction with ERK5. The actual site phosphorylated by ERK5 might be determined by either different cellular cofactors, which can align the kinase domain of ERK5 with different phosphorylation sites of these three MEF proteins; or that the more subtle variations in the amino acid sequences of each of the MEFs can contribute to the precise site phosphorylated by ERK5.

In this regard, a conserved p38-docking domain was found in both MEF2A and MEF2C. In comparison, the docking domain of MEF2D is significantly different and has been considered inaccessible by p38 [65], suggesting that the recognition and interaction of ERK5 with MEF family members is less stringent compared to p38. The other possibility is that ERK5 recognises domains in these MEFs distinct from that utilised by p38. Moreover, ERK5 has been shown to physically interact with amino acids found within the DNA binding domain of MEF2A and MEF2C, which is also a putative hint in direction of different regulation [99].

Phosphorylation of MEF2 proteins via two MAPK cascades suggests a matching role in regulation. However it is still unclear whether p38 and ERK5 act in concert to induce *c-jun* expression, since p38 is activated by stress and inflammation but not by mitogens [43, 51, 57].

The functional consequence of ERK5 association with MEF2D was demonstrated by Kasler et al. A C-terminal region of ERK5 from amino acids 664-789 is a potent transcriptional activation domain and the MEF2-interacting domain could be mapped to amino acids 440-501 [101]. Both domains are necessary for co-activation of MEF2D by ERK5. This interaction is dependent on calcium influx *in vivo* and inhibited in vitro by the calcium-sensitive MEF2 repressor Cabin 1. ERK5 mediated activation of MEF2D leads to upregulation of the endogenous Nur 77 promoter. Nur 77 is a member of the orphan steroid receptor family [102] required for the apoptosis of immature T cells in response to antigen receptor signals.

II-2. MEF2 family of transcription factors

II-2.1. Structure of the MEF2 family members

Members of the MEF2 family of transcription factors were originally described as having a DNA-binding activity and recognising an A/T-rich element promoter of the muscle creatine kinase enhancer [103]. This element is present in the control regions of almost all-skeletal and cardiac muscle genes. In vertebrates four different *mef2* genes have been identified [104] referred to as *mef2a, -b, -c,* and *–d* (Figure 8), which are located on different chromosomes [105].

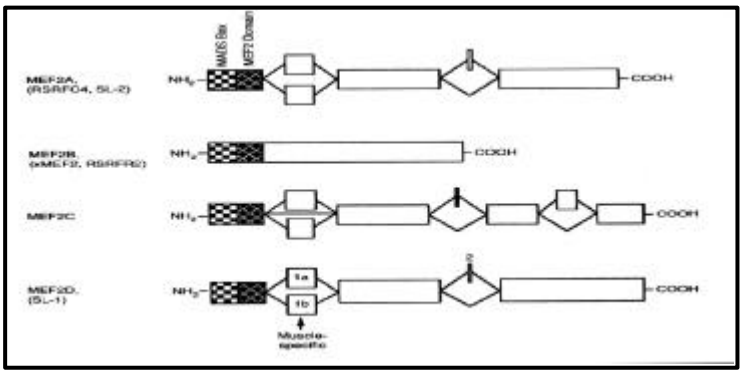

Fig. 8 Schematic representation of the MEF2 proteins. The MADS (chequered) and MEF2 (crosshatched) domains are indicated at the amino-terminus of each protein. Alternative exons are shown.

MEF2 factors belong to the superfamily of transcription factors that have a highly conserved region termed MADS domain (MCM1-agamous-and-deficiens-serum response factor).

The MADS-box is a 57-amino acid motif located at the extreme N-terminus of the MEF2 factors (Figure 7). This motif serves as minimal DNA-binding domain. An adjacent 29-amino acid extension referred to, as MEF2 domain, unique for MEF2 factors, is required for high affinity DNA binding and dimerization [106]. In addition the MEF2 domain is important for interactions

with accessory factors. MEF2 factors can homo- and heterodimerise but they cannot interact with other MADS-box factors. This suggests that amino acid residues mediating such an interaction are not present outside of the MEF family [104].

Amino acid homology is about 95% within the MADS-box and MEF-domains, whereas a certain divergence is observed at the C-terminus of the family members. Homology in the conserved regions is constrained between the species.

The MADS-box and MEF2 domains are necessary and sufficient for DNA binding, but they lack transcriptional activity of their own [104].

MEF2 proteins preferentially bind to the consensus sequence YTA$(A/T)_4$TAR, whereas the rest of the MADS-family, like SRF, bind effectively to CC$(A/T)_6$GG, known also as CArG-box. The DNA-binding of MEF2A, -C and -D is increased in comparison to MEF2B [105, 107, 108].

One interesting feature of MEF2 proteins is their ability to recognise and discriminate between A/T related binding sites. It appeared that their capacity depends on three basic amino acids at positions 1, 11 and 15 within the MADS-box [105]. Sequences outside of the MADS-box were also shown to influence DNA-binding specificity [109]. Flanking MEF2 site nucleotides were increasing the binding activity [107, 110, 111].

II-2.2. Tissue and cell type distribution of MEF2 proteins

During the embryogenesis *mef2* transcripts are highly enriched in developing muscle lineages. *mef2c* is the first of the *mef2* genes to be expressed in the mesodermal precursors giving rise to the heart [112]. The transcripts of the other *mef2* genes are expressed shortly afterwards in the developing myocardium. *mef2* gene expression is detected in developing skeletal and smooth muscle lineages concomitant with the activation of their differentiation programs. High levels of

MEF proteins are detectable in developing muscle lineages during mammalian embryogenesis [113].

In vitro MEF2D has been reported to be expressed in proliferating myoblasts prior to onset of differentiation [114]. MEF2A proteins are detectable when the cells enter the differentiation pathway, and MEF2C is expressed in the late differentiation program.

In addition MEF2 proteins are expressed at high levels in the developing central nervous system [115-118].

Targeted deletion of the MEF2C gene revealed its importance not only for early cardiogenesis [119] but also resulted in vascular anomalies characterised by extreme variability in lumen size and defects in remodelling [120]. The defects were accompanied by a notable reduction in angiopoietin 1, and VEGF mRNA production by the myocardium, indicating that MEF2C is required for myocardial expression of these important endothelial-directed cytokines and thus for correct endocardial morphogenesis.

II-2.3. Regulation of MEF2 activity

MEF2 expression and activity is a subject of a tight regulation. It occurs at transcriptional, translational and post-translational level and thus provides a possibility to direct the activity of the family members at multiple time points.

II-2.3.1. Transcriptional control

No cis-acting elements have been described so far. One reason is that the 5' non-coding region of vertebrate *mef2* is very large. The picture becomes even more complicated because of the existence of multiple alternatively spliced exons and large introns. However a certain progress towards defining the regulatory cis-elements that regulates *mef2* transcription in *Drosophila* has

been made. The *D-mef2* gene contains a dozen independent enhancers within the 12 kb upstream of the gene, each of which directs transcription in a unique temporospatial pattern throughout the development [104].

II-2.3.2. Translational control

The transcripts for MEF2A, -B, and -D are ubiquitously expressed, whereas those for MEF2C show expression in skeletal muscle, heart and brain [105, 107, 121-123]. The corresponding proteins however, are largely restricted to muscle and neural tissue [107, 115, 124]. Such a disparity between the MEF2 protein and mRNA level could be explained with the fact that in the 3' untranslated region of MEF2A transcripts an evolutionary conserved region may play a regulatory role [125]. This region might inhibit the expression of the transcript in non-muscle cells and inhibition becomes released upon muscle differentiation. Other studies performed in vascular smooth muscle cells show that protein level of MEF2A proteins is increased upon serum stimulation without corresponding change in mRNA levels [126].

II-2.3.3. Post- translational control

II-2.3.3.1. Regulation by protein interactions

In many non-muscle cell lines such as HeLa and NIH3T3 fibroblasts, MEF2 binding activity is comparable to that of C_2C_{12} myotubes, but MEF2 lacks transcriptional activity in those cells [127]. The fact that in muscle cells the DNA-binding complexes are composed of MEF2A homodimers, whereas in non-muscle cells they exist as MEF2A:MEF2D heterodimers, might be an explanation for this observation. It is likely that transcriptional silencing of the heteromeric complex depends on the presence of MEF2D. It is questionable, how MEF2D, which itself has a transcriptional activation domain, serves under certain conditions as transcriptional silencer; and whether such an activity might be regulated.

II-2.3.3.2. Regulation of the MEF2 proteins via phosphorylation

Phosphorylation plays a very important role in regulation and activation of MEF2 family members. For example the MADS-box in vivo is highly phosphorylated [128], which suggests that further phosphorylation in this region rather leads to conformational changes, than to activation.

Activation through phosphorylation is observed by the members of the mitogen-activated protein (MAP) kinase family. It is well studied that MAPK signalling enhances transcriptional activity of MEF transcription factors in a variety of cell types. As an example phosphorylation by p38 takes place at the C-terminal transactivation domain of MEF2. p38 kinase was shown in several studies to regulate the MEF2 transcriptional activity and thus to control different processes (see p 9). Phosphorylation at position Ser 387 was mediated by p38 and ERK5 [57, 93], and was critical for activation. Interestingly this serine is conserved between MEF2A and MEF2C, but not in MEF2D.

II-2.3.4. Control of MEF2 transcriptional activity by interaction with myogenic basic Helix-Loop-Helix proteins

The question whether MEF2 proteins possess muscle-inducing activity on their own was a subject of controversy. Kaushal et al. reported that MEF2 factors could activate myogenesis in transfected 10T1/2 and NIH3T3 fibroblasts with efficiency comparable to that of the myogenic bHLH proteins [129]. In contrast other groups [107, 130, 131] found MEF2 factors incapable of activating myogenesis alone, instead, these factors co-operate with myogenic bHLH proteins. MEF2 proteins augment the myogenic activity of myogenic bHLH proteins. In the case of MyoD this is dependent on 2 'myogenic' amino acids in the basic region of that protein [104, 129, 130].

Very imporatant step in induction of myogenesis is the transition of the myoblasts to low-mitogen medium. It is because the activity of the myogenic bHLH proteins, such as MyoD, is suppressed when myoblasts are cultured in high serum medium [132-136]

Combinatorial interaction between MEF2 and bHLH proteins is the key mechanism in controlling skeletal myogenesis. This synergy could be followed on the level of native promoters. MRF4 and desmin genes appear to synergistically upregulated by cotransfection of myogenic bHLH proteins and MEF2 proteins [137, 138]. In several studies it has been shown that synergistic activation is also mediated by co-ordinate positioning of MEF2 binding sites and E-boxes often positioned with precise spacing to allow both factors to bind DNA simultaneously [139, 140]

The direct interaction between MEF2 and bHLH proteins is mediated by the MADS-box and the bHLH regions of these factors. MEF2 factors can only interact with heterodimers of myogenic bHLH proteins and E-proteins but not with homodimers of E-proteins [141]. A well-established model describes how MEF2 proteins can co-operate with myogenic bHLH proteins and synergistically activate muscle-specific transcription (Figure 9).

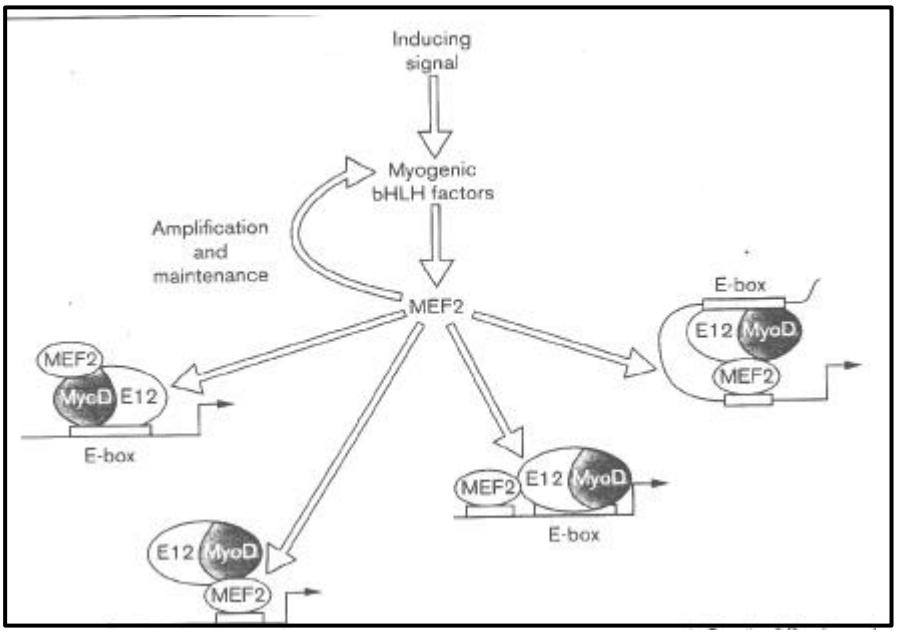

Fig. 9 A model for the co-operative activation of muscle specific transcription by myogenic bHLH and MEF2 factors. Myogenic bHLH factors are induced in muscle precursor cell in response to inducing signals. Myogenic bHLH factors up-regulate expression of MEF2 factors, which feedback on myogenic bHLH genes to amplify and maintain their expression in committed muscle cells. Myogenic bHLH and MEF2 factors collaborate to induce muscle structural genes by binding directly to muscle specific control regions or through indirect interactions.

II-2.3.5. A two-step model for transcriptional activation of muscle specific genes

The function of myogenic bHLH proteins such as MyoD is kept under thight regulation and activation of muscle gene transcription occurs only in cells cultured at low-mitogen medium. The activity of myogenic bHLH proteins is suppressed by high serum medium or peptide growth factors. In many cases even forced expression of MyoD cannot override the inhibition caused by growth factors. This result shows that a post-translational mechanism/s controls the activity of muscle specific gene regulators. For example it has been shown that phosphorylation plays very important role upregulation of the transcriptional activity of MEF2 proteins and myogenic bHLH (see this work pp. 27 and 38).

It is thought that the activated MEF2 and bHLH proteins ‘perform’ the myogenic program via their DNA-binding domains and that mutation or substitution of the 'myogenic residues' in the basic domain of bHLH proteins is sufficient to abolish this process [142-147]. Recent studies indicated that the MEF2-bHLH interaction alone is insufficient to activate myogenic transcription and myogenesis. Studies performed with a class of MyoD mutants that support MEF2 interaction but are not able to activate myogenesis indicate that initiation of myogenesis requires activation signals by both activated MEF and myogenic bHLH proteins. These signals have to be transmitted to the transcriptional machinery via myogenic bHLH factor bound to DNA [147]. These studies suggest a two-step model for transcriptional synergy. In step one (*cofactor binding*), MEF2 and myogenic bHLH proteins by recruitment to their DNA-binding domains. In step two (*transmission of the activation signal*), the transcriptional activation domains of these factors contact with the basal transcriptional machinery to establish an active transcriptional complex. Transmission of the activation signal occurs through the DNA-bound cofactor and in the case of myogenic bHLH proteins is dependent on the presence of the ‘myogenic’ amino acids alanine and threonine in the basic region.

II-3. Superfamily of bHLH proteins

MRFs (myogenic regulatory factors) belong to the superfamily of basic helix-loop-helix (bHLH) proteins, which is composed of proteins involved in a wide array of developmental processes including cellular proliferation and differentiation [148].

bHLH proteins have been grouped into four classes based on partner choice, DNA-binding ability, tissue distribution and transcriptional activity:

A. *Class A* or E-proteins are ubiquitously expressed and serve as partners for at least two other classes [149-151]. Members of this class are E12, E47, HEB and E2-2 or *Drosophila melanogaster*-daughterless [148].

B. *Class B* or tissue-specific bHLH proteins pair with class A bHLH proteins to produce heterodimers and function as transcriptional activators [152-154]. This class includes MyoD, myogenin, Neuro D, and members of the achaete-scute complex in *Drosophila melanogaster* [148].

C. *Id proteins* – are inhibitory proteins, which lack a DNA-binding basic region and form non DNA-binding complexes with *class A* and some of the *class B* proteins [149, 155].

D. *Hairy and Enhancer-of-split* are proteins that also act as repressors of bHLH proteins and can bind to DNA and inhibit promoter activity [156-158].

II-3.1. Myogenic regulatory factors (MRFs)

II-3.1.1. Structure of MRFs

The MRFs group consists of four members (Table 1): Myf5, MyoD, myogenin and MRF4, all of which are expressed in skeletal muscles. One of the unique features of MRFs is that ectopic

expression in fibroblasts or other non-muscle cells leads to initiation of the myogenic program and convert those cells to myogenic derivatives [159, 160].

Human	Mouse/rat	Chick/quail	Toad
myf3	myoD	CMD1/qmf1	XmyoD
myf4	myogenin	myogenin/qmf2	Xmyogenin
myf5	myf5	myf5/qmf3	Xmyf5
myf6	herculin/MRF4	herculin	XMRf4

Electric ray	Sea urchin	Fruit fly	Nematode
MyoD	SUM1	nautilus	hlh1
myogenin			
myf5			

Table 1. The MyoD gene family. The four myogenic regulatory factor genes are conserved amongst vertebrates.

In muscle cells MRFs form heterodimers with ubiquitously expressed bHLH proteins, known as E-proteins, and activate gene transcription by binding to a consensus DNA-site: CANNTG (also called E-box) in muscle specific regulatory elements. Although these E-boxes all share the CANNTG-motif, differences in the binding site preferences of various bHLH heterodimers for different CANNTG-motif containing sequences may play an important role in subtle regulation of the expression of different muscle genes [161, 162].

The basic domain (Figure 10) mediates DNA-binding [143, 145, 161, 163], whereas the HLH domain enables protein dimerization between different members of the bHLH family [146, 164, 165].

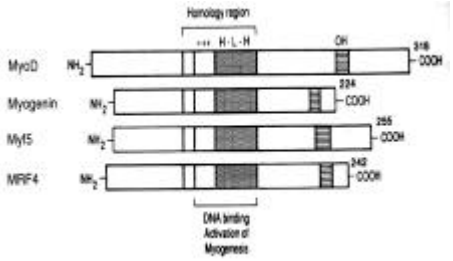

Fig. 10 Structural comparison of the mammalian bHLH family of myogenic regulatory factors. The region of homology that encompasses the basic (+++) and HLH domains is shaded. The region that is sufficient for myogenesis is indicated at the bottom. A serine/threonine-rich homology region (OH) is indicated by stripes. The number of the aminoacids is shown at the end of each box.

The segment containing 12-amino acids is necessary and sufficient for DNA binding. In E-proteins 8 of these amino acids are conserved. In the centre of the segment two amino acids are required for activation of the myogenic program - namely A and T (Figure 11).

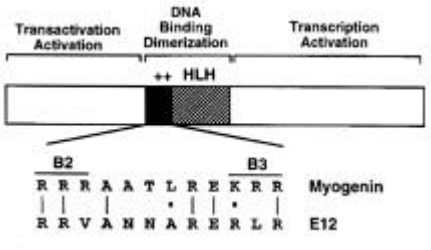

Fig. 11 Functional domains of myogenin. Myogenin contains transcription activation domain near the N- and C-termini, which are required for activation of muscle gene transcription. The bHLH region is necessary and sufficient for dimerization and DNA binding. The sequences of myogenin and E12 are shown. The alanine and threonine residues in the centre of the myogenin basic region are required for muscle gene activation but not for DNA binding. B2 and B3 denote the second and third clusters of amino acids in the DNA binding domain

Interestingly these residues are not responsible for DNA binding [142, 143]. When replaced by asparagine, the corresponding mutants retain their ability of binding DNA, but are not any longer capable of inducing muscle gene activation. On the other hand if the asparagines in the E12 basic region are replaced alanine-threonine this substitution leads E12 capable of inducing myogenesis. The same effect is observed when the aspartic acid at the junction of the basic region and of the helix-1 of E12 is replaced by lysine found in at the same position in the basic region of myogenic factors [145].

Residues in the basic region required for myogenesis are conserved in all known myogenic factors through the species ranging from Drosophila [166, 167] and sea urchin [168] to humans [169]. They are not found at the corresponding positions of the more than 50 known bHLH proteins [170].

II-4. Myogenic differentiation

II-4.1. Mouse knock-out studies and *in vitro* myogenic differentiation

Myocyte differentiation is a multicellular process simultaneous with cell fusion and formation of myotubes. Mouse knockout studies (Summarised in Table 2.) revealed at least two distinct developmental functions for MRFs [171]:

a) Myf5 and MyoD act as determination genes

b) Myogenin functions as a differentiation gene

MyoD and Myf5 double knock-out mice fail to produce or sustain a significant population of myoblasts, whereas myogenin null mutants are highly deficient in muscle differentiation although they have a certain amount of myoblast precursor cells.

Major muscle phenotype of MRFs-knockout mice					
Developmental stage	MyoD-/-	Myf5-/-	Myf5-/- MyoD-/-	Myogenin-/-	MRF4-/-
Myotome before E9.5	Normal	No myocytes no myogenin	-	Anatomically wild-type	Reduced early myotomal myogenesis
Myotome after E10.5	Normal	'Recovering' myotome	-	Severe differentiation defect	'Recovering' myotome
New-born	Grossly normal increased Myf5	Grossly normal	Few cells increased fat	Severe differentiation defect	Mainly normal

Table 2 Major muscle phenotype of MRFs-knock-out mice.

In all known skeletal lineages, myf5/ MyoD expression is followed by upregulation of the expression of myogenin and MEF2 at the beginning of terminal cytodifferentiation. MEF2 expression is upregulated differentially starting with the expression of MEF2C and MEF2A, B and D appearing later [108, 112]. MRF4 is the last MRF to be activated in most muscle types.

II-4.2. Regulation of myogenesis during development

Skeletal muscle develops from the myotomes of the somites. The myotome is a postmitotic compartment that grows by continuos addition of new MRFs-expressing myoblasts coming from a precursor pool that is thought to be located in dermomyotome.

During the process of myogenic differentiation the sequential activation of at least one member of each of the three functional subgroups: determination MRFs, differentiation MRFs and MEF2 factors is a prerequisite. Current data suggest that at least two separate pathways induce different determination MRFs in distinct domains of somites [172]. Thus the neural tube and the dorsal ectoderm induce expression of myf5 and MyoD respectively in the developing somite. As a result myotome is composed of two individual separate trunk muscle cell sublineages. Myf-5 initiated cells are the earliest to be induced and later they contribute to the epaxial lineage that give rise to the muscles of the deep back. The cell group marked by the expression of MyoD and begins to appear later is probably responsible for producing hypaxial muscles of the body wall.

Limb muscle arise from a pool of cells that originate from ventrolateral region of the somite. These cells delaminate and migrate to developing limbs. They are Pax3, Lbx1, c-Met and Msx1 positive. Upon arrival expression of Pax3 becomes downregulated and the expression of MRFs is upregulated.

II-4.3. Extracellular hints that mediate myogenic differentiation

Several factors are expressed in axial and lateral regions of the developing embryo, which are important for somite formation and the determination of cell lineages [173] (Figure 12). Axial structures, such as the neural tube and notochord, provide signals necessary for epaxial myogenic determination [174-177]. By contrast, the hypaxial myogenic lineage is dependent upon signals originating from the lateral plate mesoderm and dorsal ectoderm [172, 178, 179]. Factors

secreted from these structures include sonic hedgehog (Shh), Wnts, transforming growth factor-beta (TGF-beta)-like molecules, fibroblast growth factors (FGFs) and the bone morphogenic proteins (BMPs).

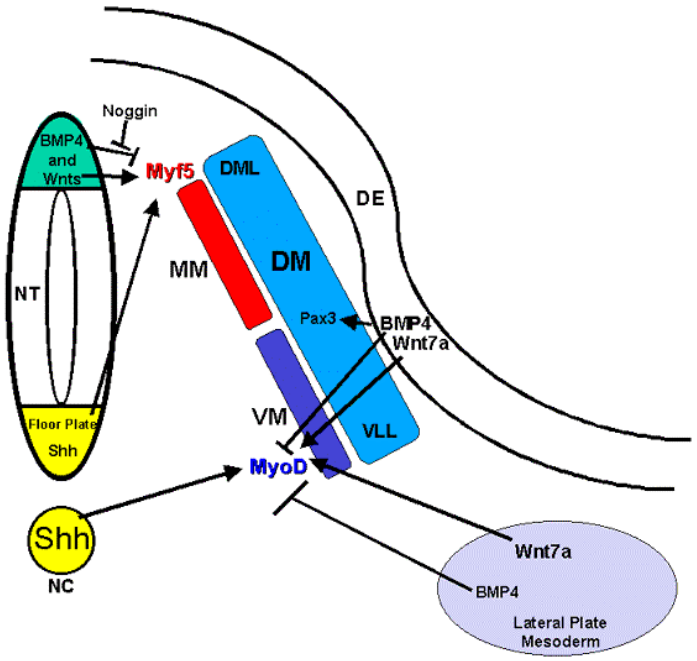

Fig.12 Extracellular growth factors, which are important for myotomal development. Sonic hedgehog is secreted by both notochord and floor plate which serves to induce Myf5 expression. Wnts, in particular Wnt1, secreted from the dorsal neural tube similarly induce Myf5 expression in the epaxial myotome. By contrast, Wnt7a secreted from the dorsal ectoderm induces MyoD expression in the ventral myotome. BMP4 secreted from the dorsal ectoderm and lateral plate mesoderm is important for repressing MRFs activation and maintaining Pax3 expression in cells of the dermomyotome and the migrating precursor population in the VLL. Both dorsal neural tube and the DML secrete noggin, inhibiting the repressive effects of BMP4 on myogenesis. DML=dorsomedial lip; VLL=ventrolateral lip; DE=dorsal ectoderm; NT=neural tube; NC=notocord; MM=medial myotome; VM=ventral myotome.

All these factors regulate myogenic determination and differentiation.

Another important mechanism during development is cell-cell contact. It contributes to the formation of distinct cell types. The transmembrane proteins of the Notch-Delta/Jagged signalling pathway are involved with cell contact signalling in myogenic regulation [180]. Upon interaction of a Notch expressing cell with a Delta/Jagged expressing cell, the intracellular portion of Notch is cleaved, translocates to the nucleus and suppresses differentiation. Overexpression of the cytoplasmic portion of Notch represses myogenesis [181]. During

development, Notch2 is expressed in cells of the DML, which lie juxtaposed to Delta expressing cells in the developing somite [182]. This suggests that Notch2 suppress myogenic commitment prior to cells extending beneath the dermomyotome.

II-4.4. The role of signal transduction in myogenic differentiation

Treatment of cells with growth factors and cytokines leads to the activation of several intracellular kinase pathways which ultimately lead to changes in gene expression, cell survival and cellular morphology (for review see [183]). Many distinct mechanisms have been elucidated to explain how signalling cascades, activated by growth factors, are able to repress or stimulate the myogenic program.

II-4.4.1. Protein kinase C (PKC)

Protein kinase C (PKC) activity is increased in response to mitogenic stimulation. Overexpression of activated PKC represses MRFs-mediated transcription of muscle-specific promoter elements and terminal differentiation. Transcriptional activation and DNA-binding are regulated by the direct phosphorylation of a threonine residue in the basic domain of myogenin [184]. Although this threonine residue is conserved in all four MRFs, PKC phosphorylation is specific for myogenin suggesting that PKC-mediated regulation of myogenesis involves other pathways [185].

II-4.4.2. The MAPK signalling pathway

Binding of ligands to cell-surface receptors initiates a cascade of events, which leads to the activation of p21Ras. Overexpression of activated p21Ras in 10T1/2 mouse fibroblasts inhibits MRFs-mediated differentiation without altering DNA-binding or the inherent transcriptional activation properties of the MRFs [186]. Interestingly, inhibition of MEK and Rac/Rho kinase pathways, which are activated by Ras, do not rescue myogenesis suggesting these pathways are

not involved in regulating terminal differentiation [187]. However, more recent studies demonstrate that inhibition of the MEK signalling pathway alleviates the repressive effects of FGF on myoblast differentiation [188]. Furthermore, overexpression of the MAPK phosphatase, MKP-1, which is normally upregulated during differentiation, is important for inhibiting ERK activity and permitting differentiation [189]. It should be noted that later stages of differentiation require MKP-1 downregulation for myoblast fusion and myotube formation [189]. Taken together, it is clear that activation of Raf/MEK/ERK signalling is required for transmitting growth signals and decreases in MAPK activity is required for myogenesis to proceed.

II-4.4.3. The Phosphatidylinositol 3-kinase (PI3'K) pathway

Insulin-like growth factors (IGFs) are known to positively regulate myogenesis. IGF stimulation leads to activation of phosphatidylinositol 3-kinase (PI3'K). Expression of dominant negative forms of PI3'K or inhibition of PI3'K activity using synthetic inhibitors results in a block of IGF-mediated differentiation [190-192]. When IGF signalling is blocked, cells maintain high levels of Id proteins and are unable to upregulate p21Cip1 for cell cycle withdrawal [190]. Conversely, expression of activated PI3'K is able to induce differentiation suggesting a direct role for PI3'K in myogenesis [192].

The lipid products resulting from stimulation of PI3'K activity serve to activate protein kinase B (PKB/Akt). During differentiation, PKB expression is upregulated and its activity is important for myocyte survival [193]. Surprisingly, activated PKB is able to phosphorylate Raf, rendering the Raf/MEK/MAPK pathway inactive [194]. Although this inhibition is important during differentiation, overexpression of activated PKB does not force differentiation under growth conditions suggesting the involvement of mediators that are specifically expressed at the onset of myogenic differentiation [195].

In many cell lines, the absence of extracellular growth factor stimulation leads to apoptosis indicating that growth factor induced pathways are essential for cell survival.

Although platelet-derived growth factor (PDGF) and IGF elicit opposite responses in myoblast cell lines, either factor on its own is sufficient to prevent apoptosis [38]. Two distinct pathways are utilised indicating that cell survival can be mediated by separate mechanisms [38]. It is surprising that myoblasts stimulated with PDGF, which is mitogenic, produce a transient PKB activation and prolonged ERK activation. By contrast, IGF leads to transient ERK activation and prolonged PKB activity suggesting that the decision to proliferate is dependent upon the length of time that the MAPK pathway is active [38].

MEF2 proteins are positively regulated by both p38 stress-activated and MEK5/ERK5 kinase pathways [57, 65, 66, 93]. The finding that MEF2 factors represent downstream targets of these pathways suggests that activation of MEF2 transcriptional activity is an important step during myogenesis. Indeed, overexpression of p38 isoforms or upstream activators stimulates myogenesis [67, 196]. It is interesting to note that the gamma isoform of p38 (SAPK3-beta/ERK6) is highly expressed in skeletal muscle. Although expression of this kinase is upregulated upon differentiation, it does not appear to phosphorylate MEF2 proteins and therefore its function remains unclear [196, 197].

Aim of the project

Many negative regulators of myogenesis have been identified and described such as Id, Twist, oncogenic Ras, viral proteins E1A, and simian virus T40 antigen [198].

Not much is known about the positive regulation of myogenic differentiation; the link between the receptor on the plasma membrane and transcription factors governing the process or the kinases that trans-mediate positive signals.

There are several hints suggesting a potential role of MEK5/ERK5 MAPK pathway in myogenesis. One obvious indication is the expression pattern, which shows abundance in heart and skeletal muscle [79]. On the other hand phosphorylation of the members of the MEF2 family by ERK5 upon stimulation is another very important clue [93, 99, 100], which prompted the examination of the direct function of the kinase cascade during muscle cell differentiation.

As a model system the mouse myoblast C_2C_{12} cell line was chosen, which proved to be a very useful tool in studying the process of myogenic differentiation [199]. To analyse and describe the role of MEK5/ERK5 MAPK pathway several experiments were designed including promoter reporter studies, which will be described in detail in the following chapter.

To study myogenic differentiation, which is a multi-cellular phenomenon that co-incides with cell fusion and myotube formation, the approach of choice was to create cell lines stably expressing components which interfere with MEK5/ERK5 signalling.

III. RESULTS

III-1. ERK5 becomes strongly activated upon induction of differentiation in C_2C_{12} myoblasts

To explore ERK5 involvement in the process of myogenic differentiation the mouse C_2C_{12} skeletal muscle cell line was analysed, which have been proven to be an excellent model system for defining mechanisms, involved in the ´decision´ to grow or to differentiate [199]. These cells proliferate in medium containing 10% foetal bovine serum (FBS) and can be induced to differentiate after withdrawal of mitogens by addition of 2% horse serum to the medium [199] (Figure 3-1).

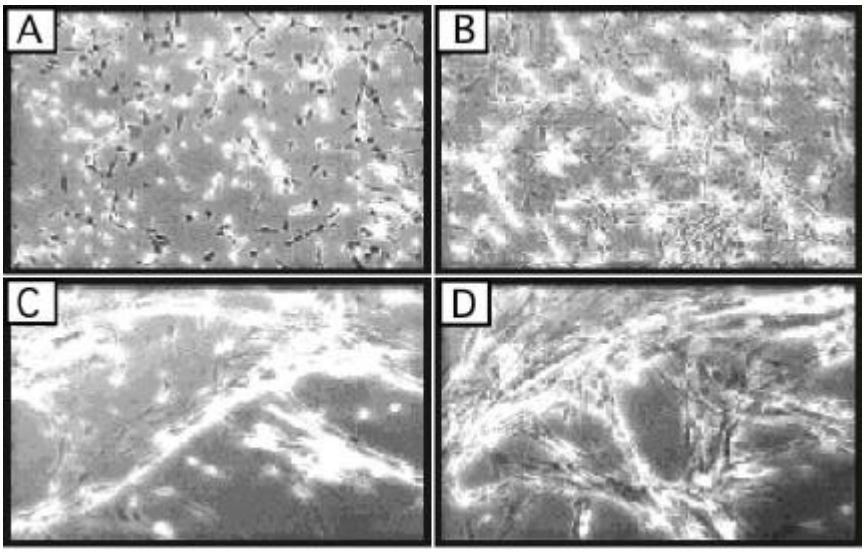

Fig. 3-1 Induction of myogenic differentiation in C_2C_{12} myoblast upon differentiation conditions. C_2C_{12} myoblasts were plated at a density of $2.5X10^5$ on 10cm dishes in DMEM/10%FBS. 24h later cells were shifted to DMEM/2%HS to induce differentiation: **A.** Control in growth medium; **B.** 1 day in Differentiation medium; **C.** 3 days in Differentiation medium; **D.** 5 days in Differentiation medium

Under such conditions a strong activation of endogenous ERK5 as early as 5min post stimulation and persisting up to 60 min could be observed. (Figure 3-2).

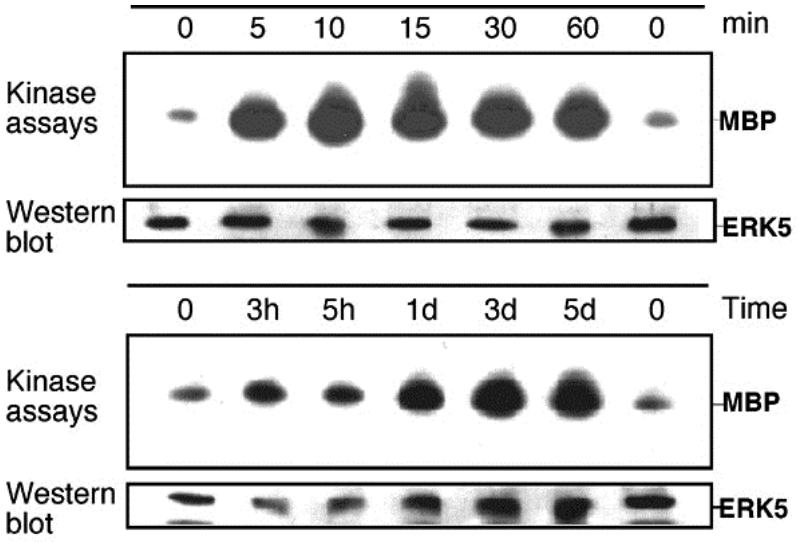

Fig. 3-2 ERK5 is activated upon differentiation in C_2C_{12} cells. C_2C_{12} myoblasts were plated as indicated and induced to differentiate. Cell lysates were prepared at different time points as indicated. ERK5 was immunoprecipitated from the lysates using a polyclonal goat anti-ERK5 antiserum. ERK5 activity was measured by an immune complex kinase assay using MBP as a substrate. Equal loading of kinases was assessed by immunoblotting with an ERK5-specific antiserum.

Similar activation kinetics were observed when a transfected flag-tagged version of ERK5 was analysed (Figure 3-3A). Interestingly, a sustained upregulation of ERK5 activity was also observed at later stages of differentiation (days 1-5) concomitant with the formation of myotubes. This suggests a regulatory role in both early and later stages of differentiation. ERK5 activity is cell density potentiated in C_2C_{12} cells grown to confluence in 10% FBS DMEM (Figure 3-3C). In contrast, in a control, ERK5 is only moderately active in cells cultured in growth medium with 10% FBS (Figure 3-3B).

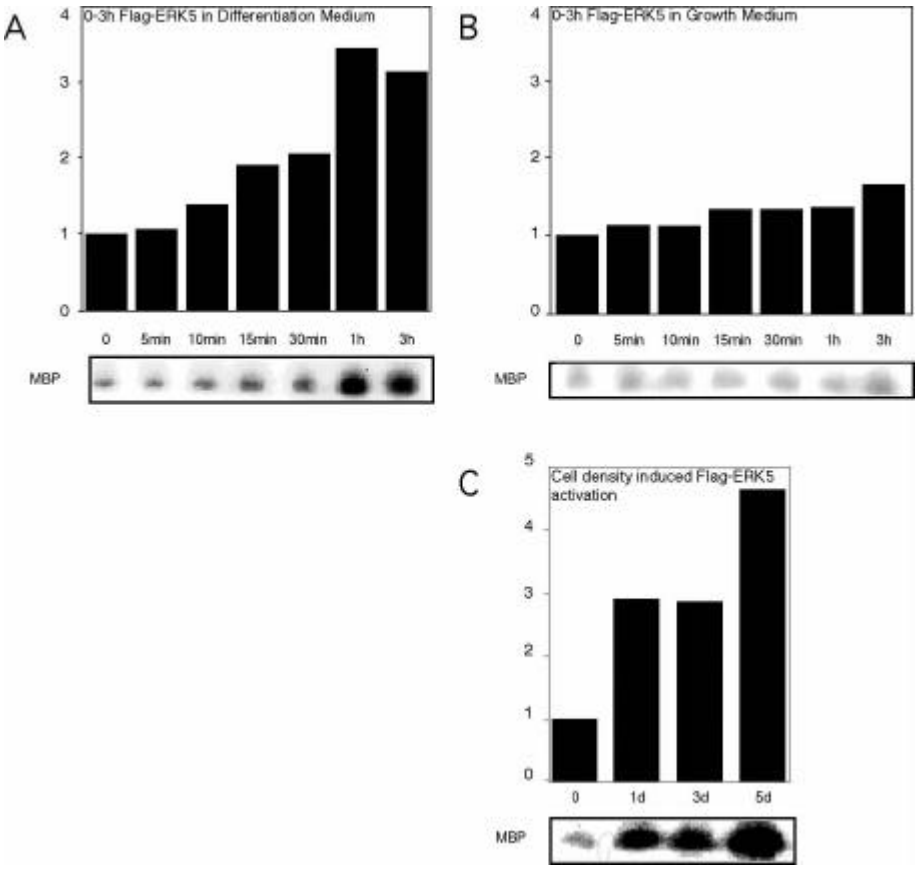

Fig. 3-3 Overexpressed Flag-ERK5 behaves similar to endogenous and its activity is only moderately influenced by growth factors in C_2C_{12} cells. ERK5 activation is cell density mediated. C_2C_{12} myoblasts were plated at density $2{,}5x10^5$ one-day prior transfection on 10-cm plates. Cells were transfected with 5µg Flag-tagged ERK5 overnight. On the next day cells were washed 2 times with PBS and induced to differentiate (A) or shifted to 10% FBS DMEM (B) and (C) for different time points as indicated. ERK5 was immunoprecipitated from cell lysates using a polyclonal goat anti-Flag antiserum. ERK5 activity was measured by an immune complex kinase assay using MBP as a substrate.

III-2. Transactivation of differentiation-specific promoters is dependent on ERK5 activity

III-2.1. MEK5/ERK5 expression upregulates the activity of the p21 promoter under differentiation conditions

If ERK5 activation has functional consequences during muscle cell differentiation than overexpression of ERK5 or MEK5 should interfere with the promoter transactivation of individual differentiation-specific genes. One of the early markers highly upregulated during muscle development is the cyclin-dependent kinase inhibitor p21/Cip-1 [200]. Transient transfection studies on C_2C_{12} cells showed that overexpression of either wild type ERK5 (ERK5WT) or a constitutively active form of the upstream activator MEK5 (MEK5D) results in a moderate transactivation of the p21 promoter (Figure 3-4, lanes 2 and 3). Promoter activity was further potentiated by cotransfection of MEK5D with ERK5WT (Figure 3-4, lane 5). This effect was indeed mediated by ERK5 since promoter activity was reduced to basal levels if MEK5D was cotransfected with a kinase-inactive version of ERK5 (ERK5AEF) (Figure 3-4, lane 6).

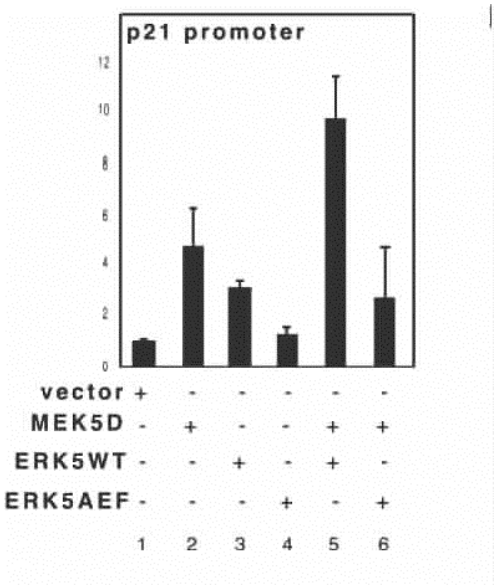

Fig. 3-4 MEK5 and ERK5 induce the transactivation of the p21 promoter upon muscle differentiation. A p21 promoter-luciferase construct was cotransfected with expression vectors encoding constitutively active MEK5 (MEK5D), wildtype ERK5 (ERK5WT), and/or a dominant negative version of ERK5 (ERK5AEF), as indicated. 20h after transfection cells were shifted to DMEM/2%HS for another 24h and than harvested to measure luciferase activity as described. Fold-induction is the ratio of luciferase activity in kinase transfected cells versus cells transfected with the empty vectors.

III-2.2. A 4xE-box containing promoter is strongly transactivated by MEK5/ERK5 when muscle differentiation is induced

One feature shared by the p21 promoter and promoter regions of muscle genes is the presence of E-box motifs [201, 202]. Here myocyte-specific-bHLH proteins like MyoD or myogenin bind as heterodimers with ubiquitously expressed bHLH proteins, such as E47, to form transcriptionaly active complexes [164]. To assess whether MEK5/ERK5 affect E-box-dependent transcription a construct with the luciferase gene under the control of a four E-box-containing promoter (4xE-box) [203] was used. This promoter was moderately transactivated by coexpression of MEK5D and ERK5WT (Figure 3-5, lane 6), however, both kinases imparted a strong synergistic effect on the activity of coexpressed MyoD (Figure 3-5, lane 11). This transactivation was again abolished if ERK5WT was replaced by kinase inactive ERK5AEF (Figure 3-5, lane 12) indicating the requirement for an active ERK5 for promoter induction.

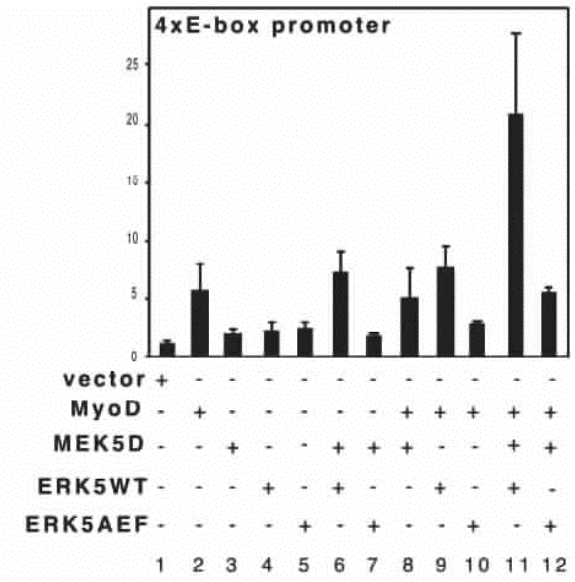

Fig. 3-5 MEK5 and ERK5 induce the transactivation of promoter motifs upregulated upon muscle differentiation. A 4xE-box containing promoter luciferase construct was cotransfected with expression vectors encoding constitutively active MEK5 (MEK5D), wildtype ERK5 (ERK5WT), a dominant negative version of ERK5 (ERK5AEF), and MyoD as indicated. 20h after transfection cells were shifted to DMEM/2%HS for another 24h and than harvested to measure luciferase activity as described. Fold-induction is the ratio of luciferase activity in kinase transfected cells versus cells transfected with the empty vectors.

Similar results were obtained for a 10T1/2 fibroblast cell line, which can also be committed to differentiate and exhibit a myogenic phenotype (Figure 3-6).

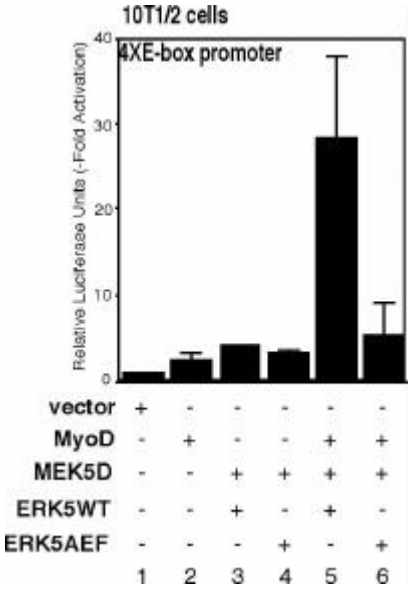

Fig. 3-6 MEK5 and ERK5 induce the transactivation of promoter motifs upregulated upon muscle differentiation. A 4xE-box containing promoter luciferase construct was cotransfected with expression vectors encoding constitutively active MEK5 (MEK5D), wildtype ERK5 (ERK5WT), a dominant negative version of ERK5 (ERK5AEF), and MyoD as indicated. 20h after transfection cells were shifted to DMEM/2%HS for another 24h and than harvested to measure luciferase activity as described. Fold-induction is the ratio of luciferase activity in kinase transfected cells versus cells transfected with the empty vectors.

III-2.3. MLC1A promoter activity is induced by the MEK5/ ERK5 signalling pathway under differentiation specific conditions

To show that the effects mediated by the MEK5/ERK5 pathway also act on promoters of muscle specific genes a *myosin light chain 1A gene* (MLC1A) promoter was used. MLC1A is one of the earliest sarcomeric muscle genes expressed in skeletal muscle [204] and is transcribed during the early stages of differentiation of skeletal muscle cell lines [205]. The promoter region of the MLC1A gene contains several E-boxes [206] and accordingly, ERK5 coexpression with MEK5D and MyoD results in an enhanced promoter activity in a similar fashion to that observed with the 4xE-box promoter (Figure 3-7, lane 10).

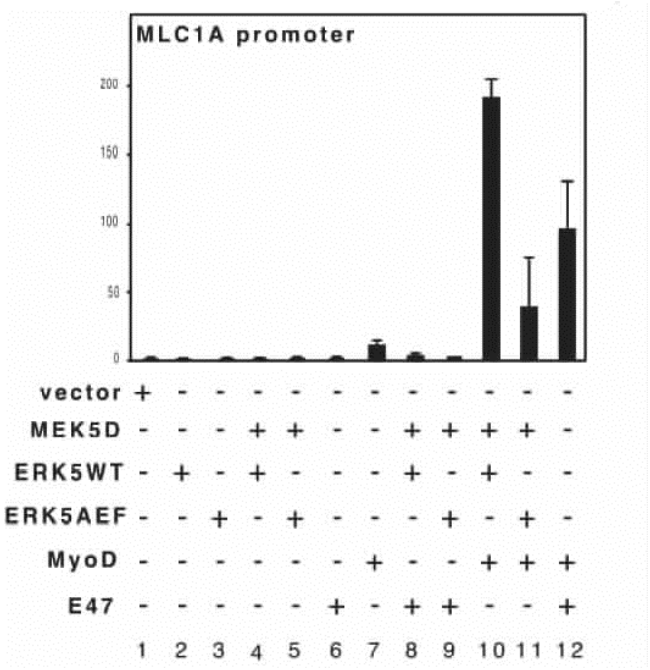

Fig. 3-7 MEK5 and ERK5 transactivate the MLC1A promoter upon muscle differentiation. A MLC1A promoter luciferase construct was cotransfected with expression vectors encoding constitutively active MEK5 (MEK5D), wildtype ERK5 (ERK5WT), a dominant negative version of ERK5 (ERK5AEF), and MyoD or E47 as indicated. 20h after transfection cells were shifted to DMEM/2%HS for another 24h and than harvested to measure luciferase activity as described. Fold-induction is the ratio of luciferase activity in kinase transfected cells versus cells transfected with the empty vectors.

Interestingly, cotransfection of MEK5D and ERK5WT with E47, another bHLH protein, which also plays a role in muscle-specific gene transcription, did not result in any significant promoter transactivation (Figure 3-7, lane 8) although E47 readily co-operates with MyoD in this process (Figure 3-7, lane 12). This suggests a functional synergism between the MEK5/ERK5 pathway and MyoD in muscle-specific gene transcription.

III-3. Activation of the MEK5/ERK5 signalling pathway under differentiation conditions suggests a synergism between MEF2C and MyoD on transcriptional level

III-3.1. MEK5/ERK differentiation mediated activation leads to upregulation of the transcriptional activity of MEF2C

It is known that molecular basis of myogenic regulation is governed by the co-operation between myogenic bHLH and MEF2 proteins. Thus it is likely that the synergism between MyoD and MEF2C might be influenced by ERK5 [93] (Figure 3-8B). To analyse whether ERK5 activation under differentiation conditions also leads to an enhanced transactivation of MEF2C a Gal4-MEF2C fusion construct was used, which was strongly activated in the presence of MEK5D and ERK5 (Figure 3-8A).

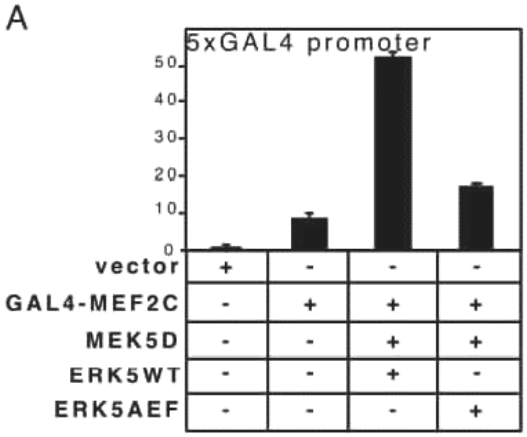

Fig. 3-8A MEF2C is activated by ERK5 in C_2C_{12} cells under differentiation conditions. C_2C_{12} cells were transfected with a Gal4-MEF2C fusion construct and transactivation of MEF2C under differentiation conditions induced by coexpressed MEK5D, ERK5WT and ERK5AEF was monitored by luciferase expression from a 5xGal4-luciferase construct.

III-3.2. Activated ERK5 is able to phosphorylate MyoD *in vitro*

Interestingly, ERK5 phosphorylates not only MEF2C but also MyoD *in vitro* (Figure 3-8B). Although this is no *in vivo* evidence yet it may be the basis of another regulatory level controlled by ERK5. This could explain the strong synergism between ERK5 and MyoD on muscle specific promoters.

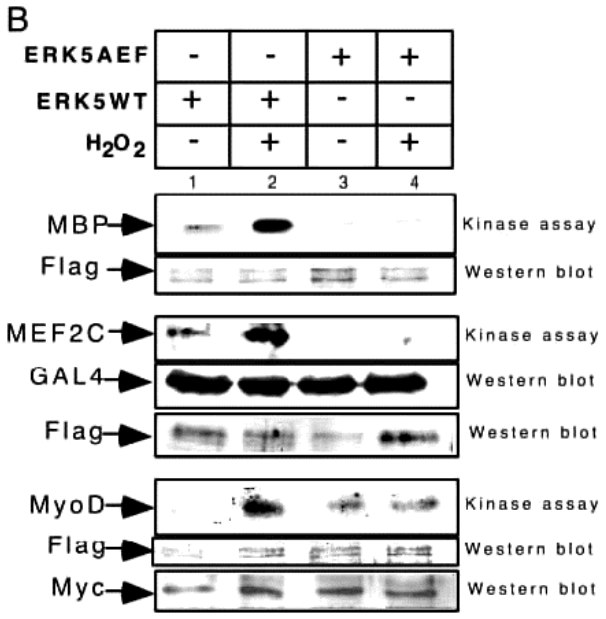

Fig. 3-8B MEF2C is activated by ERK5 in C_2C_{12} cells under differentiation conditions. Flag-ERK5WT and flag-ERK5AEF were immunoprecipitated with an anti-flag antiserum from transfected 293 cells which were either left untreated or treated with H_2O_2 (200µM, 60 min) for activation of the kinase. Immunocomplexes were incubated in a kinase reaction with either MBP (upper panel) or Gal4-MEF2C (middle panel) and Myc-MyoD (lower panel) which were immunopurified from transfected 293 lysates with anti-Gal4 and anti-Myc antibodies.

In summary, the MEK5/ERK5 pathway appears to enhance myogenic gene transcription by a mechanism dependent on the presence of myogenic activators, such as MyoD and MEF2C.

III-4. Block of ERK5 expression leads to inhibition of myogenic differentiation

III-4.1. Generation of stably expressing C_2C_{12} cell lines using retroviral infection

The process of myocyte differentiation is a multicellular phenomenon coincident with cell fusion and formation of myotubes [200]. To monitor the function of ERK5 in a pool of differentiating myoblasts cell lines overexpressing an extra copy of ERK5WT or ERK5AEF as well as a cell line transcribing an antisense RNA to block the expression of endogenous kinase were generated (Figure 3-9A).

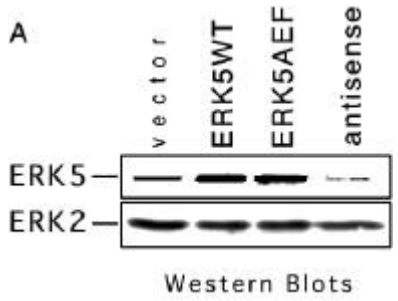

Fig. 3-9A Block of ERK5 expression results in the inhibition of myogenic differentiation. ERK5 expression in stably transduced C_2C_{12} cell lines harbouring empty vector, ERK5WT, ERK5AEF or antisense-ERK5.

Stable expression in C_2C_{12} cells was achieved by infection with different transducing retroviruses. Transduced cells were identified by expression of GFP from an IRES element within the same mRNA.

III-4.2. Myogenic differentiation is impaired when the expression of ERK5 is inhibited

After seeding, the different cell lines were allowed to proliferate in growth medium for 24h and then induced to differentiate. The increase in densities of parental cells and vector, ERK5WT or ERK5-antisense expressing cell lines was not significantly different after 24h incubation in

growth medium. This indicates that the proliferative potential is not affected upon positive or negative interference with ERK5 expression (data not shown). However, upon induction of differentiation the typical morphological alterations of differentiating myocytes, e.g. parallel orientation, cell fusion and formation of myotubes (Figure 3-9B, upper panels) were completely suppressed if ERK5 expression was blocked by an antisense-RNA (Figure 3-9B, lower panel).

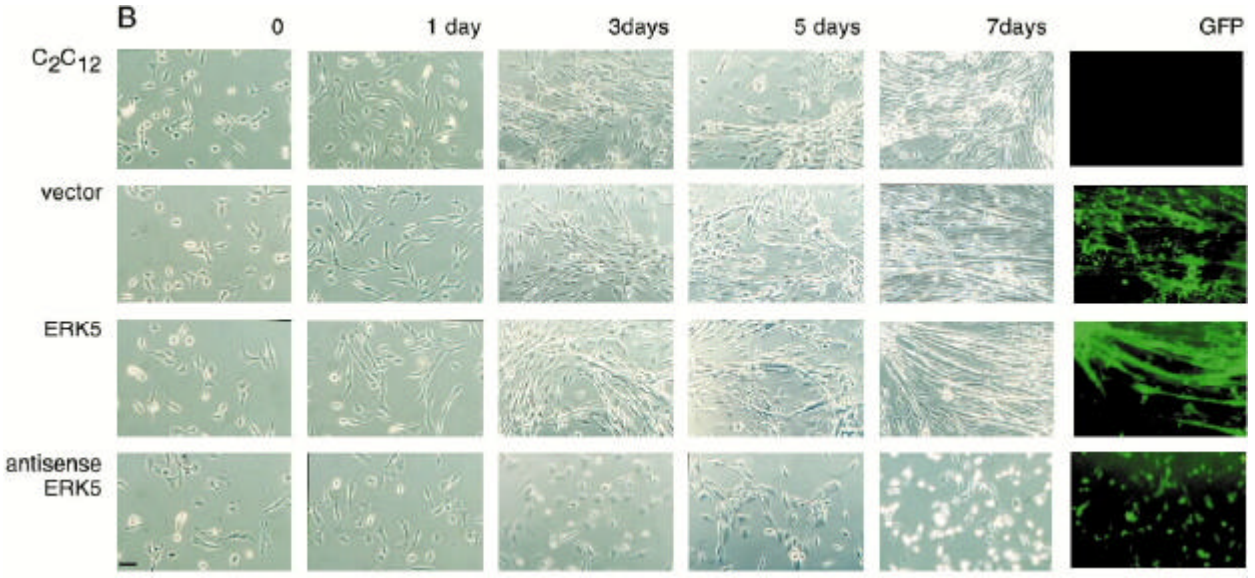

Fig. 3-9B Block of ERK5 expression results in the inhibition of myogenic differentiation. Cells were seeded at a density $2X10^6$ cells per 10cm in DMEM/10%FBS were shifted 24h later to DMEM/2%HS to induce differentiation. Pictures at indicated time points correspond to distinct stages of differentiation. Expression of GFP (last column) is indicative of successfully transduced cells. Bar equals to 65µm.

III-4.3. Blockage of the endogenous ERK5 leads to inhibition of the expression of myogenic proteins

Since inhibition of the endogenous kinase led to phenotypic impairments in the myogenic differentiation, the question, which remained to be addressed, is whether this is reflected on the expression levels of myogenic differentiation markers. Induced expression of differentiation specific genes such as MyoD, myogenin or the cell cycle inhibitor p21 was nearly abolished (Figure 3-9C). Although the effects on morphology were not that pronounced in the ERK5AEF

cell line (data not shown) the expression levels of muscle-specific genes was also strongly reduced (Figure 3-9C). Impaired expression of these differentiation markers persists throughout the observation period of 7 days (data not shown), indicating that inhibition of ERK5 expression does not simply delay the differentiation process.

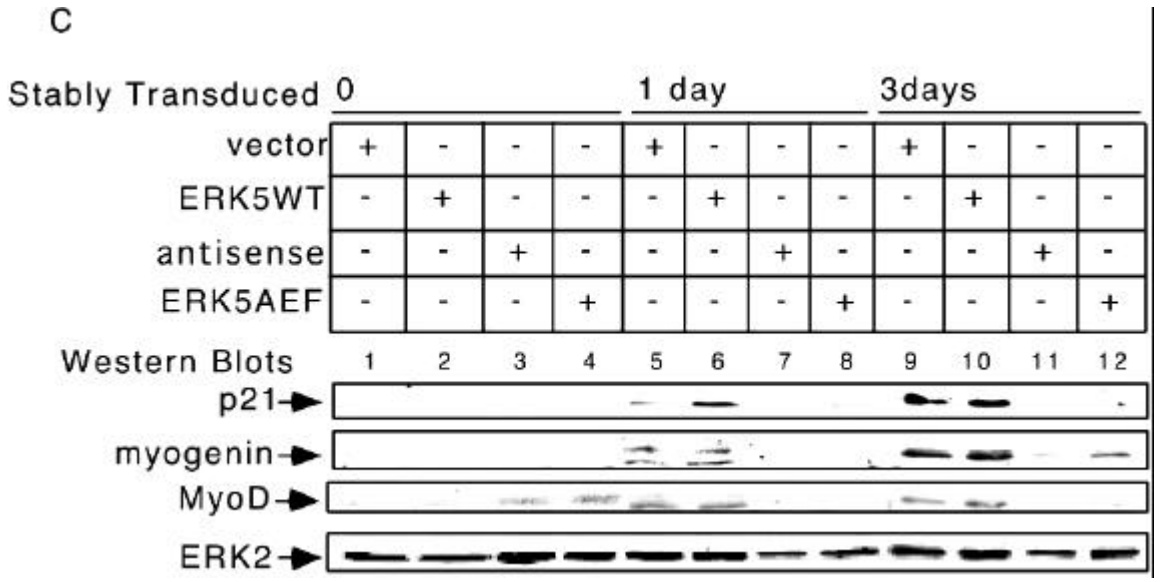

Fig. 3-9C Block of ERK5 expression results in the inhibition of myogenic differentiation. Cells were lysed in TLB after 1 and 3 days post differentiation induction and protein lysates were separated on SDS PAGE gels. After subsequent immunoblotting blots were analysed for p21, MyoD and myogenin with the respective antisera described in the Methods section. Equal protein loading of the different samples was controlled with an anti-ERK2 antiserum.

These experiments show that insufficient levels of ERK5 render C_2C_{12} cells incapable of upregulating differentiation-specific genes such as MyoD, p21/Cip-1 and myogenin, and thereby blocking morphological differentiation (Figure 3-9B).

III-4.4. ERK5 overexpression is sufficient for the commitment of C_2C_{12} cells to differentiate

To examine whether ERK5 is not only required but also sufficient to induce differentiation early passages of the different cell lines were cultured for 3 days in growth medium. Interestingly, under these conditions the morphology of ERK5WT overexpressing cells resemble the

phenotypic changes observed in early stages of the myocyte differentiation process (Figure 3-10A).

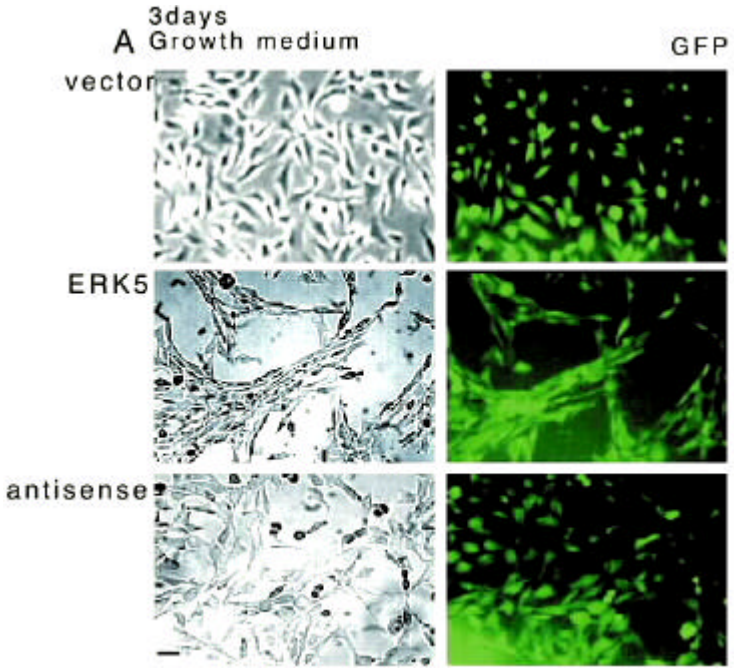

Fig. 3-10A ERK5WT overexpression is sufficient for the commitment of muscle cells to differentiate. The stably transduced C_2C_{12} cell lines indicated were grown for 3 days in DMEM/10%FBS and were then examined morphologically

Expression of MyoD was not altered (figure 3-10B) and myogenin was not induced under these conditions, however, expression of p21 was upregulated (Figure 3-10B). This may give rise to the induction of a differentiation prone phenotype. These data demonstrate that ERK5WT overexpression is sufficient for the commitment of C_2C_{12} cells to differentiate.

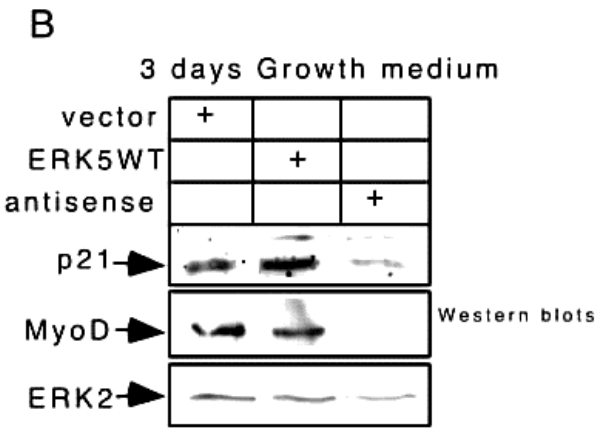

Fig.3-10B ERK5WT overexpression is sufficient for the commitment of muscle cells to differentiate. Cells shown in **Fig.3-9A** were lysed and cell lysates were subjected to SDS PAGE. After blotting, proteins were detected with antibodies against p21, MyoD, myogenin (not shown) and ERK2 as a loading control.

The MEK5/ERK5 pathway was previously shown to be involved in the stress and mitogenic responses of cells [90, 93, 207, 208]. The present studies demonstrate for the first time a biological role for ERK5 in the process of muscle cell differentiation. Thus, the MEK5/ERK5 cascade in muscle cells may resemble the classical mitogenic MAP kinase cascade, which has similarly been shown to be important for both proliferation and differentiation in certain cell types [40, 209].

IV. Discussion

The main goal of this study was to define a function for a newly discovered signalling cascade termed the MEK5/ERK5 pathway. Since the expression pattern of these kinases showed a predominant expression in muscle [79] it was worth investigating a putative physiological function that the kinases might play in this tissue. Another hint in this direction is that upon stimulation ERK5 moves to the nucleus, where it phosphorylates members of the MEF2 transcription factors, which are one of the key players in myogenic differentiation process.

IV-1. ERK5 becomes strongly activated upon induction of differentiation in C_2C_{12} myoblasts

The biphasic activation of ERK5 upon serum removal (Figure 3-2) suggests a regulatory role in the early stage of 'commitment' as well as at later time periods – when the terminal differentiation and irreversible cell cycle withdrawal take place. This phosphorylation is more distinguishable compared to the phosphorylation status of the kinase when growth factors are present. The basal phosphorylation level under 'normal' growth medium suggests a regulatory event – as long as growth factors are present, the kinase is kept 'silent', but once the differentiation conditions take over, the kinase becomes rapidly phosphorylated and ready to activate myogenic program. Certain discrepancy exists because ERK5 was shown to be activated upon growth factors like EGF or NGF. It is likely, however, that those observations are rather cell type dependent, than to be a general observation. Moreover Mody et al. suggested a negative effect on activation of MEK5/ERK5 pathway by classical MAPK cascades exists, since PD184352 (a selective ERK1/ERK2 inhibitor) at concentration of 1-2μM, prolonged the 'activation' of MEK5/ERK5 by EGF [231]. Last but not least in this regard, in the beginning

ERK5 was reported as kinase that becomes activated by stress inducers like peroxide and sorbitol.

It is worth noting that the activation is sustained, slowly but steady declining, with a double peak, suggesting a difference between ERK5 and p38, which is also implicated in myogenic program, and its activation was only observed in confluent cells one day after induction of differentiation [68].

So far it is not known, which signal triggers ERK5 in muscle cells placed in differentiation medium. Interestingly the kinase was activated by cell-to-cell contact when cells were grown 3-5 days in normal growth medium (Figure 3-3C). This is likely to be by a mechanism/s that silences the activity of mitogen dependent factor/s and leads to activation of ERK5, as it has been shown that CDO, a transmembrane Robo-related cell surface protein, mediates myogenic differentiation [210]. The role of cell-to-cell-contact in ERK5 activation, however, seems to be not the predominant mechanism, since ERK5 is efficiently activated in subconfluent myoblasts placed under differentiation conditions (Figure 3-2). And again the activity of p38 is more efficiently activated in confluent muscle cells placed in DM than in subconfluent myocytes placed under the same contions [68]

It is also very important to note that ERK5 overexpression in C_2C_{12} cells induces differentiation prone phenotype (Figure3-10B).

IV-2. Transactivation of differentially-specific promoter is dependent on ERK5 functional activity

Next step in examination of functional consequences of ERK5 activation during muscle cell differentiation is the analysis of individual differentiation specific promoters. The conclusion to

be withdrawn is whether and how overexpression of ERK5 or MEK5 will interfere with the transactivation of promoters active during the process of muscle differentiation.

IV-2.1. MEK5/ERK5 upregulate the activity of endogenous human p21 promoter under differentiation conditions

The commitment to differentiation is mediated by forced overexpression of cyclin-dependent kinase inhibitor p21/Cip-1. P21, a member of the family of cyclin-dependent inhibitors (CKI), negatively regulates the progression through the G1 phase by modulating the activity of cdk2 and cdk4 that belong to the family of cyclin-dependent kinases (cdk's). It has been shown that p53 tumour suppresser gene product, directly upregulates p21 on transcriptional level after DNA damage. MyoD upregulates p21promoter as muscle cells differentiate. DNA-binding elements for both these transcription factors exist in the p21 promoter [201]. The expression of p21 is induced in a number of ways. For example sustained activation of MAPK kinase cascades and especially Ras leads to forced p21 expression [232-234]. P21/Cip-1 leads to a cell cycle withdrawal [211], resistance to apoptosis [212]; and to induction of myogenin, which is necessary for terminal differentiation [213]. As one of the early markers highly up-regulated during muscle development, p21/Cip-1 arrests cells in G1 phase [200, 201]. ERK5 mediated upregulation of p21 promoter suggests a direct role of the pathway during the cell cycle withdrawal as an ultimate step in execution of the myogenic program.

The similar effects on the p21 promoter activity, of the constitutively active MEK5D, propose equity of the effects mediated by the dual-specificity kinase and the nuclear shuttle kinase.

IV-2.2. Expression of 4xE-box containing promoter is upregulated by MEK5/ERK5 when muscle differentiation is induced

Several examples exist, which suggest a connection between p21 and MyoD expression and vice versa. For example in fibroblasts, p21 is expressed at low levels whereas forced expression of MyoD results in a dramatic upregulation of p21 [214]; forced expression of p21 in proliferating myoblasts allows MyoD to activate muscle transcription [215, 216]. P21 is also upregulated during differentiation of myoblasts in culture and in muscle cells during embryogenesis [160, 215, 217]. In addition one feature which is shared by the p21 promoter and promoter regions of other muscle-specific genes is the presence of E-box motifs [201, 202]. Here myocyte-specific-bHLH proteins like MyoD or myogenin bind as heterodimers with ubiquitously expressed bHLH proteins, such as E47, to form transcriptionaly active complexes [164].

As expected MEK5 and ERK5 were able to affect E-box-dependent transcription. Although the promoter was marginally transactivated by co-expression of MEK5D and ERK5WT (Figure 3-5, lane 6), these kinases imparted a strong synergistic effect on the activity of co-expressed MyoD (Figure 3-5, lane 11). The transactivation was again abolished if ERK5WT was replaced by kinase inactive ERK5AEF (Figure 3-5, lane 12).

Similar results were obtained with the 10T1/2 fibroblast cell line (Figure 3-6), which exhibits a myogenic phenotype when MRF are ectopically expressed [159, 160].

These observations show that ERK5 is an important player in transactivation of the reporter construct containing putative MyoD binding sites, and that the effect is mediated by the kinase is direct.

A novel finding is that these results suggest a strong synergism between MEK5/ERK5 signalling pathway and p21 on one hand, and MyoD on the other, at level of upregulation of transcriptional activity.

IV-2.3. MLC1A is induced by MEK5/ ERK5 signaling pathway under differentiation specific conditions

Data from 'natural' promoters like the promoter of myosin light chain 1A (MLC1A) gene, which is expressed in skeletal muscle [204], shows that ERK5 mediates enhanced promoter activity in a similar fashion to that observed with the 4xE-box promoter (Figure 3-7). This supports the observation that ERK5 is indeed required for bHLH proteins mediated transcription and furthermore that transcriptional activity may be up- or downregulated depending on the presence of active or inactive kinase, respectively. Interestingly, co-transfection of MEK5D and ERK5WT with another bHLH protein, E47, which also plays a role in muscle-specific gene transcription, did not result in any significant promoter transactivation (Figure 3-7, lane 11) although E47 co-operates with MyoD in this process (Figure 3-7, lane 12). This observation has several aspects to consider. First, certain specificity on the level of interaction and activation is suggested, because ERK5 does not upregulate promoter activity in the presence of E47. E47 doesn't possess a Ser/ Thr rich region like MyoD, which might be one of the reasons why there is no certain cross talk between ERK5 and E47. On the other hand activity of E47 has shown to be negatively regulated by 3pk linking E47 function to MAPK-activated protein kinases [203]. Second, it is likely that putative effects mediated by ERK5 over the MRFs promote heteromerisation versus homomerisation, thus favouring transcriptional active heterodimers, which has been shown to be responsible for the execution of myogenic differentiation program. Alternatively beside its effect on heteromerisation of MRF with class A bHLH proteins, ERK5 may bring together heterodimers together with activated MEF2 proteins serving as a bridge.

IV-3. Activation of MEK5/ERK5 signaling pathway under differentiation conditions suggests a synergism between MEF2C and MyoD on transcriptional level

IV-3.1. MEK5/ERK differentiation mediated activation leads to upregulation of the transcriptional activity of MEF2C

One of the urgent questions to be addressed was to determine the mechanism underlying MEK5/ERK5 mediated muscle differentiation. Could MEK5/ERK5 modulate the activity of MEF2 transcription factors in favouring transcriptional active complexes on DNA level with bHLH proteins? Is ERK5 capable of activating MEF2 transcription factors upon induction of differentiation process?

As seen in Figure 3-8A, under differentiation conditions, ERK5 strongly enhances transcriptional activity of MEF2C fused to yeast GAL4 DNA-binding domain, measured on 5xGAL4 binding-site promoter. This transactivation is greater than that observed when MyoD was transfected together with GAL4-MEF2C (data not shown). Enhanced transcriptional activity of GAL4-MEF2C is most likely due to direct or indirect phosphorylation by ERK5 upon induction of differentiation. ERK5 phosphorylates MEF2C at position Ser 387 in the TAD. Of potential interest is to examine where is the phosphorylation site responsible for the ultimate activation of MEF2C, and whether activation of MEF2 proteins in certain cell types would correlate with certain level of phosphorylation. For example the simultaneous phosphorylation of the MEF2C on three residues (T293, T300, and S387) in the transactivation domain is important for MEF2C activation in nonmuscle cells (e.g. lymphoid cells) [57]. Wu et al. showed that p38 phosphorylates MEF2C preferentially on T293 and this is critical for MEF2C activation. However this neither affected MEF2C-MyoD interactions in mammalian-two-hybrid, nor did the

selective mutation of T293 to A have any influence on MyoD-MEF2C functional synergism [68].

Of certain interest would be also to investigate how ERK5 would modulate the activity of MyoD.

IV-3.2. Activated ERK5 is able to phosphorylate MyoD *in vitro*

MyoD and MRF4, both members of the MRF family, exist in vivo as phosphoproteins and contain multiple consensus phosphorylation sites [218]. Studies suggest that enhancement of MyoD activity is regulated to a certain extent through phosphorylation. Phosphorylation affects DNA-binding of E-proteins/MRF heteromers versus homomers [219]. Furthermore phosphorylation of MyoD on S237 by Mos [220] positively regulates MyoD increasing its activity.

In vitro kinase assays results revealed that MyoD is phosphorylated by ERK5 to an extent similar to that observed with MEF2C. This could partly explain the strong synergism between MyoD and ERK5 on the level of muscle specific promoters.

Since the C-terminal part of ERK5 is transcriptionaly active the mechanism might involve a transcriptional mega-complex, one part of which is ERK5 that turns the muscle differentiation 'on'. However at what point ERK5 plays its role in mediating positive signals to the myogenic transcriptional machinery still remains to be addressed.

IV-4. Block of ERK5 expression leads to inhibition of myogenic differentiation

To further clarify a role of ERK5 in muscle differentiation and whether it is sufficient to induce this process or required for maintaining the proper execution of the process, the following experiments were designed.

IV-4.1. Generation of stably expressing C_2C_{12} cell lines

To further establish a role of ERK5 in muscle differentiation C_2C_{12} cells containing stably integrated ERK5WT or the ERK5AEF mutant genes were produced. Several things deserve to be mentioned. First the overexpression is not vast, showing a reasonable ratio of 3:1 in comparison endogenous protein in the vector control. Second the antisense approach used in these studies is specific and challenges only the expression of the endogenous ERK5, but not of the homologue ERK2. Third the resulting phenotype is not a matter of clonal selection and is not dependent on the possible integration of the retroviral construct in a locus genes pivotal for muscle differentiation, because a pool of independent stably transduced cells was used.

IV-4.2. Myogenic differentiation is blocked when the expression of ERK5 is inhibited

After seeding, the different cell lines were allowed to proliferate in growth medium for 24h and then induced to differentiate. Cells were subconfluent to avoid the influence of contact induced differentiation. The typical morphological alterations of differentiating myocytes, e.g. parallel orientation, cell fusion and formation of myotubes (Figure 3-9B, upper panels) were completely suppressed in the cell lines in which ERK5 expression was blocked by an antisense-RNA (Figure 3-9B, lower panel).

IV-4.3. Blockade of the endogenous ERK5 leads to inhibition on the level of myogenic markers

The inhibition of the endogenous kinase expression leads to a severe phenotype during differentiation. The effect of this functional blockade can be traced back to the level of expression of myogenic differentiation markers. Accordingly, induced expression of differentiation specific genes such as MyoD, myogenin or p21 is nearly inhibited (Figure 3-9C). The effects, which ERK5AEF has on the expression level of muscle-specific genes, result in strong reduction (Figure 3-9C). Although the influence over morphology is not that pronounced (data not shown), probably because of the presence of the intact endogenous kinase, which can rescue the antisense caused phenotype.

The observed markers showed impaired expression persisting throughout the observation period of 7 days, which indicates that inhibition of ERK5 expression does not simply delay the differentiation process.

The above mentioned experiments show that insufficient levels of ERK5 render C_2C_{12} cells incapable of upregulating differentiation-specific genes such as MyoD, p21/Cip-1 and myogenin, and thereby blocking morphological differentiation (Figure 3-9B).

These experiments suggest that lack of the kinase blocks the transmission of upstream positive signals to programs that await to be executed during differentiation; or that inactive kinase or lack of the kinase, renders transcriptional complexes between MEF2 and bHLH proteins incapable of driving transcription. One can imagine ERK5 as divergent point to dispatch signals to MEF2 and myogenic bHLH proteins, thus mediating the spatial and temporal equity in activating simultaneously these key players. It is admittable that ERK5 plays a role in executing the cell cycle arrest and thereby preparing the muscle cells to enter in the differentiation process.

IV-4.4. ERK5 overexpression is sufficient for the commitment of C_2C_{12} cells to differentiate

Culturing different cell lines for 3 days in growth medium, showed that under these conditions the morphology of ERK5WT overexpressing cells resemble the phenotypic changes observed in early stages of the myocyte differentiation process (Figure 3-10A).

The results obtained from expression markers show that p21 is upregulated, whereas expression of MyoD is not altered (Figure 3-10B) and myogenin is not induced under these conditions. The upregulation of the p21 expression nicely correlates with the results obtained with the transient reporter studies. How ERK5 plays on the transcriptional level is still not very clear. ERK5 mediated p21 overexpression is very likely to give rise to the induction of a differentiation prone phenotype. These data demonstrate that ERK5WT overexpression is sufficient for the commitment of C_2C_{12} cells to differentiate.

In the light of these results it is likely that ERK5 might be responsible for p21 mediated cyclin D1 inhibition and hence entering Rb-mediated cell cycle arrest during skeletal muscle differentiation. This is further supported by studies of Lassar et al., showing that Rb promotes myogenesis by inhibiting cell cycle progression and co-operating with MyoD to activate the transcriptional activation domain (TAD) of MEF2 [221]. How MyoD mediates activation of the MEF2 TAD is unclear. The process requires Rb and phosphorylation of Ser 387 in the MEF2 TAD. ERK5, which activates MEF2C via phosphorylation at this position, could serve as a molecular link. One other mean of the mechanism regulating cell cycle arrest might be a hyperphosphorylation of cyclin D1 and its subsequent degradation (summarised on Figure 4-1).

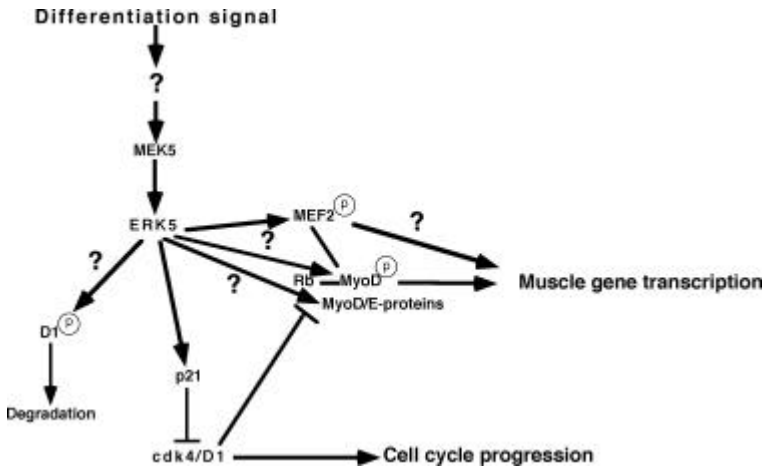

Fig.4-1 Possible modes of MEK5/ERK5 role upon induction of muscle differentiation.

The MEK5/ERK5 pathway was previously shown to be involved in the stress and mitogenic responses of cells [90, 93, 207, 208]. This study demonstrates for the first time a biological role for ERK5 in the process of muscle cell differentiation. Thus, the MEK5/ERK5 cascade in muscle cells may resemble the classical mitogenic MAP kinase cascade, which has similarly been shown to be important for both proliferation and differentiation in certain cell types [40, 209].

Finally the present results have shown that ERK5 is involved in the regulation of myogenic regulatory factors, and that certain relations between the pathway and the MRFs exist. Therefore it might be a therapeutic target e.g. stimulating the differentiation of muscle-derived tumours (rabdomyosarcomas), in which MyoD is functionally latent [222].

V. Materials and Methods

V-1. Materials

V-1.1. Instruments

Items	Companies, Type
Bacterial incubator	Heraeus B 6200
Bacterial shaker	New Brunswick Scientific innova 4330
Cell culture incubator	Köttermann
Cell culture microscope	Carl Zeiss
Culture Hood	HLB2472, BIO-FLOW Technik
Developing machine	AGFA
DNA Sequencer	ABI PRISM 373, ABI
Electrophoresis power supply	EPS600, Pharmacia
Electrophoresis unit, small	Bio-Rad Mini-Protean II
Fine scale	Scaltec SBC 21
Gel dryer	Bio-Rad Gel Dryer 583
Heat block	Liebisch, Type 2099-DA
Horizontal electrophoresis gel	MWG Biotech
Microlumat	EG&G, Berthold
Mega centrifuge	J-6B, Beckman; Megafuge 1.0 R, Heraeus; RC 5B plus, Sorval
Mini centrifuge	5417R, Eppendorf Biofuge 15, Heraeus
pH meter	Microprocessor, WTW
Phosphoimager	Fujix BAS-2000 III, Fuji, with plates BAS-MP 2040P, Fuji
Shakers	Heidolph, Unimax 2010, Edmund Bühler WS5
Scale	BP2100S, BP310S, Sartorius

Spectrophotometer	U-2000, Hitachi
Thermocycler	PE9600, Perkin Elmer
Vortex	Scientific Industries Genie-2
Water bath	GFL 1083, Amersham-Buchler

V-1.2. Reagents and general materials

Items	**Companies**
1 kb DNA ladder	Sigma
Acetyl-CoenzymA	Sigma
Acrylamide (30%)/Bisacrylamide (0,8%)	Roth
Adenosin-5'Triphosphate (ATP)	Sigma
Agarose, ultra pure	Life Technologies, Inc.
Ammonium peroxydisulfate (APS)	Sigma
Ampicillin	Sigma
Aprotinin	Roth
Bacto-Agar	Roth
Bovine serum albumin (BSA)	Sigma
Bradford-reagent	Biorad
Bromphenolblue	Sigma
Calciumchloride ($CaCl_2$)	Sigma
Chloroquine	
Circlegrow (GC)	Dianova
Deoxycholate (DOC)	Sigma
Dimethylsulfoxide (DMSO)	Sigma
Dithiothreitol (DTT)	Sigma
D-Luciferin (free acid)	Applichem
dNTP	MBI
Items	**Companies**
Ethylenediaminetetraacetic acid-disodium salt (EDTA)	Sigma

EGTA	Sigma
Ethanol	Roth
Ethidiumbromide	Life Technologies, Inc.
Glutathion-sepharose	Pharmacia
Glycerol	Sigma
Glycine	Roth
Hydrochloride (HCl)	Roth
Isoropyl-1-thio-β-D-galactopyranoside (IPTG)	Roth
Isopropanol	Merck
Leupeptin	Sigma
Magnesiumchloride	Sigma
Nitrocellulose BAS-85 membrane	Schleicher & Schüll
Ortho-nitrophenyl-β-D-galactoside (ONPG)	Applichem
Pefablock	Roth
Ponceau S	Sigma
Potassium acetate (KAc)	Sigma
Potassiumchloride (KCl)	Sigma
Potassiumdihydrophosphate (KH_2PO4)	Merck
Protein A-agarose	Roche
Protein A-peroxidase	Amersham-Buchler
Protein marker (SDS-7B)	Sigma
Radiochemicals	Amersham-Buchler
SDS ultra pure	Roth
Sodiumdihydrophosphate (NaH_2PO4)	Merck
Sodiumhydrophosphate (NaHPO4)	Merck
Sodiumhydroxide (NaOH)	Sigma
sodium morpholineethanesulfonate (Na-MES)	sigma
Sodium orthovanadate	Sigma
TEMED	Roth
Tris-(hydroxymethyl)-aminomethane (Tris)	Roth
Triton-X100	Sigma
Whatman 3MM Papier	Schleicher & Schüll

X-gal	Sigma
X-ray film	Amersham-Buchler
Xylencyanol	Roth
Yeast extract	Life Technologies, Inc.

Gerneral laboratory reagents were purchased from: Fluka Chemie, Merck, Roth, SERVA

V-1.3. Cell culture materials

Items	Companies
Lypofectamin 2000 ™	Life Technologies, Inc.
Fetal bovine serum (FBS)	PAA
L-Glutamine	Life Technologies, Inc.
Penicillin /Streptomycin	Life Technologies, Inc.
DMEM	Life Technologies, Inc.
Trypanblue	Sigma
Zeocin ™	Invitrogene

V-1.4. Enzymes

Items	Companies
Calf Intestinal Phosphatase (CIP)	New England Biolabs
Pfu Taq Polymerase	Stratagene
RNase	Roche
T4 Ligase	New England Biolabs

General restriction enzymes are purchased from Amersham-Buchler, Eurogentec, New England Biolabs, and Roche.

V-1.5. Antibody conjugates

Antibodies	**Antigens**	**References**
anti-ERK2 (C-14)	ERK2	Santa Cruz (sc-154)
anti-ERK5 (C-20)	ERK5	Santa Cruz (sc-1284)
anti-Flag M2	Flag-epitope	Kodak-IBI (Integra)
anti-GAL4BD	yeast-GAL4 Binding domain	Santa Cruz (sc-577)
anti-Myc	Myc epitope	MSZ Würzburg
anti-MyoD (C-20)	MyoD	Santa Cruz (sc-304)
anti-myogenin (M-225)	myogenin	Santa Cruz (sc-576)
anti-Mous IgG conjugated peroxidase (POD)		Amersham-Buchler
Protein-A conjugated Agarose-beads		Roche
Protein-A conjugated Peroxidase		Roche
Protein-G conjugated Agarose-beads		Roche

V-1.6. Kits

Items	**Companies**
ECL Western blotting detection reagents	Amersham
QIAEX II Gel Extraction Kit	Qiagen
QIAGEN Plasmid Kit (Midi, Maxi)	Qiagen
QIAquick PCR purification Kit	Qiagen

V-1.7. Plasmids

pcDNA3	Invitrogen
pcDNA3/BMK1WT-Flag	J.D. Lee [93]
pcDNA3/BMK1AEF-Flag	J.D. Lee [93]
pcDNA3/E47	B.Neufeld [203]
pCFG5 IEGZ	Institute for Virology, Würzburg
pCFG5 IEGZ-ERK5AEF sense	in this work
pCFG5 IEGZ-ERK5WT sense	in this work
pCFG5 IEGZ-ERK5WT antisense	in this work
pCMV5	M.Cobb [223]
pCMV5/MEK5D-HA	J.D.Lee [93]
pCMV5/MEK5WT-HA	J.D.Lee [93]
pCS2+MT	R.Rupp [224]
pCS2+MT/MyoD	in this work
pEMC11s/MyoD 5xGAL4-Luciferase	R.Rupp [225]
pGL2/p21P	Wang X.-F. [226]
pGL3/4xE-boxWT	B.Neufeld [203]
pM	Clontech
pM/Gal4BD/MEF2C	in this work

V-1.8. PCR-primers:

The PCR-primers were sythesized by MWG-Biotech AG

5'MEF2C (XmaI/SmaI restr.site)	5'-TCC*CCCGGG*GAGAAAAAAGATTC-3'
3'MEF2C (XbaI restr.site)	5'-GC*TCTAGA*TCATGTTGCCCATCC-3'

V-1.9. Bacteria strains and cell lines

Cell line	ATCC #	Species	Classification	Reference
DH5α		E.coli		
C_2C_{12}	CRL-1772	M.Musculus	myoblast	[199]
Phoenix-Eco (293T based)			packaging cell line	Nolan's lab
Phoenix-GP+Eco			packaging cell line	Nolan's lab
10T1/2	CCL-226	M.Musculus	fibroblast	[227]

V-1.10. Media:

LB (Luria-Bertani) medium

1% Bacto-tytone

1% NaCl, 0.5% Bacto-yeast extract

Adjust pH to 7.5 with NaOH and total volume to I L with H_2O

For plates, add 15 g Bacto-agar

GC (Circle-Grow) medium

4% (w/v) Circle Grow disolved in H_2O

Freeze Medium

70% Complete DMEM (10% FBS, P/S)

20% Fetal Bovine Serum

10% DMSO

2x TY medium

16 g Bacto-tryptone

10 g Bacto-yeast extract

5 g NaCl

Adjust pH to 7.4 with NaOH and total volume to 1 liter with H_2O

For plates, add 15 g Bacto-agar

V-1.11. Solutions:

Acidic KOAc (neutralization buffer)

3 M KOAc

2 M HOAc, store at 4°C

b-Gal Assay Buffer

100 mM Na-phosphate-buffer, pH7.4

10 mM KCl

1 mM $MgSO_4$

3.5 µl/ml β-mercaptoethanol

Blotting Buffer (for transferring the proteins to the nitrocellulose membrane)

39 mM Glycine

48 mM Tris

0.037% SDS

10% Methanol

Blocking Buffer

5% (w/v) of nonfat dry milk in TBST

Buffer P1 (Resuspension buffer)

50 mM Tris-HCl, pH 8.0

10 mM EDTA

10 mg/ml Rnase A

Buffer P2 (Lysis buffer)

10% SDS

200 mM NaOH

Buffer P3 (Neutralization buffer)

3 M potassium acetate, pH5.5

Buffer QBT (Equilibration buffer)

15% ethanol

0.15% Triton X-100

Buffer QC (Wash buffer)

2.0 M NaCl

50 mM MOPS, pH7.0

15% ethanol

Buffer QF (Elution buffer)

1.25 mM NaCl

50 mM Tris-HCl, pH 8.5

15% ethanol

1x CIP Buffer

50 mM NaCl

10 mM Tris-HCl

10 mM $MgCl_2$

1 mM dithiothreitol , pH7.9

10x DNA Gel Loading Buffer

40% (w/v) saccharose

0.25% bromphenolblue

0.25% xylencyanol, use as 1x solution

Kinase Buffer

10 mM $MgCl_2$

25 mM HEPES, pH 7.5

25 mM β-glycerophosphate

1 mM Sodium vanadate

0.5 mM DTT

Luciferase Assay Buffer

125 mM Na-MES, pH 7.8

125 mM Tris-HCl, pH 7.8

25 mM magnesium acetate

2 mg of ATP per ml

Luciferin Solution

1 mM D-luciferin in 5 mM KH_2PO4

Lysis Buffer (for cell culture)

50 mM Na-MES, pH 7.8

50 mM Tris-HCl, pH 7.8

10 mM DTT

2% Trion X-100

ONPG-solution

4 mg/ml ONPG in 0.5 M Na-phosphate buffer, pH7.0

Phospate-Buffered Saline (PBS)

136 mM NaCl

2.6 mM KCl

10 mM Na_2HPO_4,

1.5 mM KH_2PO_4, pH7.4

Radioimmune Precipitation (RIPA) Buffer (high stringent IP Buffer)

25 mM Tris-HCl, pH 8.0

137 mM NaCl

10 % (v/v) glycerol

0.1% SDS

0.5% (v/v) deoxycholate (DOC)

1% (v/v) Nonidet P-40

2 mMEDTA

1 mM pefablock

1 mM sodium vanadate

5 mM benzamidine

5 µg/ml aprotinin

5 µg/ml leupeptin

Running Buffer (for SDS-PAGE)

25 mM Tris

250 mM Glycine

0.1 % SDS

5x SDS-loading buffer (for SDS-PAGE)

31 mM Tris HCl, pH6.8

1% SDS

5 % Glycerin

2.5 % Mercaptoethanol

0.05 % Bromphenoblue, work at 1x solution

Sodium Tris-EDTA buffer (STE)

100 mM NaCl

10 mM Tris-HCl, pH 8.0

1 mM EDTA

TLB buffer

20 mM Tris, pH 7.4

50 mM Sodium -glycerophosphate

20 mM sodium pyrophosphated

500 mM NaCl

10 % (v/v) glycerol

0.1% Triton X-100

2 mMEDTA

1 mM pefablock

1 mM sodium orthovanadate

5 mM benzamidine

5 µg/ml aprotinin

5 µg/ml leupeptin

1x Tris-Acetate-EDTA (TAE)

40 mM Tris-HCl,

40 mM acidic acid,

2 mM EDTA; pH7.8

10x Tris-Borate-EDTA (TBE)

108 g Tris base

55 g boric acid

20 ml 0.5 M EDTA, pH 8.0

Adjust the volume to 1 liter with H_2O

10x Tris-Buffered Saline (TBS)

1 mM Tris-HCl,

150 mM NaCl

TBST

1x TBS + 0.1% Tween

V-2. Methods

The following protocols were used for all experiments unless otherwise indicated.

V-2.1. Working with bacteria

V-2.1.1. Bacterial cultures

Plasmid transformed bacteria are selected on LB plates with Ampicillin (50 µg/ml) for 24 hr. For overnight mini cultures, pick 1 colony and inoculate in LB medium with Ampicillin and shake overnight at 37°C. The culture is then used for preparing frozen glycerine cultures, plasmid DNA or fusion protein purification.

For storage of bacteria, a glycerol stock culture is prepared by growing bacteria in culture medium and measuring the OD with a photometer at a wavelength of 600 nm. When the OD reached 0.8, take 500µl bacterial culture out and add to 500 µl 80% glycerine and then mix thoroughly in a small 1.5 ml tube. This stock solution is subsequently frozen at –80°C. To start an overnight culture again, take out bacteria and hold at room temperature (RT) until surface is thawed. Pick a small amount of cells and mix into 2-5 ml culture medium and leave to grow for several hours at 37°C in a bacterial culture shaker. The frozen stock is immediately returned to the -80^{o}C.

V-2.1.2. Preparation of competent cells ($CaCl_2$ method)

Start an overnight preculture from a single colon on petri dish in 2 ml LB or 2x TY media by incubation at 37°C and shaking to aerate. The second day, inoculate 1 ml of the preculture in 100 ml fresh media and grow the culture at 37°C until OD at wavelength 650 nm of the culture

reaches 0.2 to 0.3. Cool down the culture on ice for at least 15 min. (The following handlings should be done at 4°C in pre-cooled sterile tubes). Harvest the cells by a 5-min centrifuge at 5000 g, and discard the supernatant. Resuspend the bacterial pellets thoroughly in a small volume of ice-cold 100-mM $CaCl_2$. Dilute the suspension with the $CaCl_2$ solution to a final volume of 30-40 ml, and leave on ice for 25 min with occasionally shaking. Spindown the cells as before, discard the supernatant carefully and resuspend the pellets in 5 ml glycerol/$CaCl_2$. The suspension can be aliquoted in 100 to 400 µl and stored at –70°C. The transformation efficiency of the bacteria prepared by this method should reach at least 10^6.

V-2.1.3. Transformation of competent bacteria

Thaw the competent bacteria from a desired origin on ice. Add maximum of 20ng ligated DNA or purified plasmid-DNA to 100 µl competent cells in a cold 1.5 ml microfuge tube. Mix carefully and keep on ice for 20 min or longer. Heat-shock the bacteria then at 42°C for 90 sec, add 1 ml antibiotic-free LB medium, and aerate at 37°C for 30 min. Selection of transformed bacteria is done by plating 100µl of the bacterial suspension on antibiotic containing agar plates. Only bacteria that have taken up the desired plasmids, which normally contain ampicillin resistance cassette, can grow on the agar plates. One of the colonies that grows on the plate can then be expanded in LB medium and used for DNA preparation.

V-2.2. Working with DNAs

V-2.2.1. Electrophoresis of DNA on agarose gel

Double stranded DNA fragments with lengths between 0.5 kb and 10 kb can be separated according to their lengths on agarose gels. Agarose is added to 1x TAE to obtain a final concentration between 0.7-2%. Boil the suspension in the microwave until the agarose is completely solubilised. Allow the agarose to cool down to around 50°C before adding ethidium bromide up to 0.5 µg/ml and pour into the gel apparatus. Add DNA gel loading buffer to the DNA sample and apply on the gel. Electrophorese in 1x TAE buffer at 100 volts. The DNA can be visualised under UV-light.

V-2.2.2. Isolation of plasmid DNA from Agarose (QIAEX II agarose gel extraction protocol)

This protocol is designed for the extraction of 40-bp to 50-bp DNA fragments from 0.3-2% standard agarose gels in TAE or TBE buffer. DNA molecules are adsorbed to QIAEX II silica particles in the presence of high salt. All non-nucleic acid impurities such as agarose, proteins, salts, and ethidium bromide are removed during washing steps.

Excise the desired DNA band from the agarose gel under the UV light. Weigh the gel slice and add 3 volumes of Buffer QG to 1 volume of gel for DNA fragments 100-bp-4 kb; for DNA fragments more than 4 kb, add 2 volume of QG plus 2 volumes of H_2O. Resuspend QIAEA II by vortexing for 30 sec, add 10 µl (or 30 µl) of QIAEX II to the sample containing not more than 2 µg of DNA (between 2-10 µg). Incubate at 50°C for 10 min to solubilise the agarose and bind the DNA. Mix by vortexing every 2 min to keep QIAEX II in suspension. Centrifuge the

sample for 30 sec and carefully remove supernatant with a pipette. Wash the pellet with 500 ml of Buffer QG and then twice with Buffer PE. Air-dry the pellet and elute the DNA in 10 mM Tris-HCL or H_2O and resuspend the pellet by vortexing. Incubate at RT for 5 min (or at 50°C for 5 min) for DNA fragments not more than 4 kb (for DNA fragments between 4-10 kb). Centrifuge for 30 sec and carefully pipette supernatant into a clean tube.

V-2.2.3. Purification of plasmid DNA (QIAquick PCR purification kit)

This protocol is designed to purify single- or double-stranded PCR products or DNA plasmids ranging from 100 bp to 10 kb. DNA adsorbs to the silica-membrane in the presence of high salt while contaminants pass through the column. The impurities are washed away and pure DNA is eluted with Tris buffer or H_2O.

Add 5 volume of buffer PB to 1 volume of the contaminants and mix. Place a QIAquick spin column in a 2 ml collection tube. Apply the mixed sample to the QIAquick column and centrifuge 30-60 sec. Discard flow-through and place QIAquick column back into the same collection tube. Add 0.75 ml Washing Buffer PE to column and centrifuge 30-60 sec. Discard flow-through and place QIAquick column back into the same collection tube. Centrifuge column for an additional 1 min at maximum speed. Place QIAquick column in a clean 1.5 ml microfuge tube. Add 50 µl Elution Buffer EB or H_2O to the centre of the QIAquick column and centrifuge for 1 min. Store the purified DNA at - 20°C.

V-2.2.4. Ligation of DNA fragments

Calf-intestinal-phosphatase (CIP) reaction (5' phosphorylation)

Alkaline phosphatase catalyses the removal of 5' phosphate groups from DNA, RNA and ribo- and deoxyribonucleoside triphosphates. For blunt end ligation, the 5’ phosphate group of the

vector must be removed by CIP reaction. This reaction is also used to prevent the re-ligation of the vectors. 2.5 µg of DNA fragments is phosphorylated at 37°C for 30 min in 100 µl of reaction volumes consisting of 1x CIP buffer and 1µl of phosphatase. 5 mM EDTA is then added to the reaction and incubated with the reaction at 65°C for 15 min to inactivate the enzyme. The DNA fragments are purified by phenolying and ethanol precipitation before ligation reaction.

V-2.2.5. Cohesive-end ligation

Prepare the plasmid DNA or DNA fragment by cutting it with suitable restriction enzymes, which is followed by purification. 1:3 molar ratio of vector: insert DNA fragments together with 1 µl of T4 ligase are incubated in 1x Ligation Buffer in a total volume of 20 µl for 4 hr at RT or overnight at 16°C. Heat the mixture at 65°C for 10 min to inactivate the enzyme.

V-2.2.6. Mini-preparation of plasmid DNA

Grow 3 ml overnight culture in LB, 2x TY, or GC media with 100 µg/ml ampicllin at 37°C overnight. Pellet the cells at 14,000 rpm for 1 min. Remove the supernatant and resuspend the pellets in 100 µl Buffer. Add 200 µl Buffer P2 (Lysis Buffer) and incubate at RT for 5 min. Add 150 µl ice-cold 3 M acidic KOAc (Neutralisation Buffer), mix by inverting the tubes for 6-7 times and incubate on ice for 5 min. Centrifuge at 15,000 rpm for 3 min. Transfer the supernatant to a fresh eppendorf tube and add 900 µl of pre-cooled 100% ethanol, precipitate at -70°C for 10 min. Centrifuge the pellet at 15,000 rpm for 10 min. Wash the pellet with 200 µl 70% ethanol. Air-dry the pellet and resusspend it in 30-50µl 10 mM Tris-HCl, pH 7.8.

V-2.2.7. Maxi-preparation of plasmid DNA

Grow culture in 50 ml GC media containing plasmids or recombinant plasmids overnight in a 37°C incubator with shaking at 220 rpm. Collect the bacteria and isolate DNA plasmids by using a Quiagen Plasmid Maxi Kit. This extraction method is based on Birnboim's alkali lysis principle. Resuspend the bacterial pellet in 10 ml of Buffer P1. Add 10 ml of Buffer P2, mix gently, and incubate at RT for 5 min. Add 10 ml of chilled Buffer P3, mix immediately, and incubate on ice for 20 min. Centrifuge at 4,000 rpm for 30 min at 4°C. Filter the supernatant over a prewetted, folded filter. Apply the supernatant to an equilibrated QIAGEN-tip 500 and allow it to enter the resin by gravity flow. Wash the QIAGEN-tip twice with Buffer QC. Elute DNA with 15 ml Buffer QF. These processes result in the isolation of a DNA-salt pellet, which is precipitated by 0.7 volumes (10.5 ml) of isopropanol and centrifuged further at 4000 rpm for 30 min. Washed the resulting pellet twice with 70% ethanol and air-dry at RT. The pellet is then carefully resuspended in TE buffer and used for transfection of cultured mammalian cells.

V-2.2.8. Measurement of DNA concentration

The DNA concentration is determined by using an UV spectrophotometer at wavelength of 260 nm. The absorption of 1 at 260 nm corresponds to a concentration of 50µg/ml double stranded DNA. Identity, integrity and possible purity of the DNA can be subsequently analysed on an agarose gel.

V-2.2.9. DNA Sequencing (Sanger Dideoxy Method)

DNA can be sequenced by generating fragments through the controlled interruption of enzymatic replication [228]. DNA polymerase I is used to copy a particular sequence of a single-stranded DNA. The synthesis is primered by complementary fragment, which may be obtained from a restriction enzyme digest or synthesised chemically. In addition to the four deoxyribonucleoside triphosphates (ddNTP), the incubation mixture contains a 2', 3'-dideoxy analogue of one of them. The incorporation of this analogue blocks further growth of the new chain because it lacks the 3'-hydroxyl terminus needed to form the next phosphodiester bond. A fluorescent tag is attached to the oligonucleotide primer, a differently coloured one in each of the four chain-terminating reaction mixtures. The reaction mixtures are combined and electrophoresed together. The separated bands of DNA are then detected by their fluorescence as they pass out the bottom of the tube, and the sequence of their colours directly yields the base sequence.

1. Sequencing Reaction:

The "Taq Cycle Sequencing" is performed by using "^{P}RISMTM Ready Reaction DyeDeoxyTM Terminator Cycle Sequencing Kit".

Mix the following reagents in a 0.6 ml double-snap-cap microfuge tube:

Terminator premix*	9.5 µl
DNA template	1.0 µg
Primer	10 pmol
dH_2O	Adjust the final reaction volume to 20µl

*A-Dye Terminator labelled with dichloro[R6G]
C-Dye Terminator labelled with dichloro[TAMRA]
G-Dye Terminator labelled with dichloro[R110]
T-Dye Terminator labelled with dichloro[ROX]

Place the tubes in a thermal cycler preheated to 96°C which is followed by 25 cycles of thermal cycling steps: 96°C for 15 sec; 48°C for 15 sec; 60°C for 4 min; and keep at 4°C after the reaction.

2. Removal of the excess dye terminators by using CENTRI-SEP Columns:

CENTRI-SEP Columns are designed for the fast and efficient purification of large molecules from small molecules.

Prepare the CENTRI-SEP columns according to the standard procedures (PRINCETON SEPARATIONS, INC.) Transfer the DyeDeoxy^{TM} terminator reaction mixture to the top of the gel. Carefully dispense the sample gently onto the centre of the gel bed at the top of the column without disturbing the gel surface. Place the column into the sample collection tube and place both into the rotor. Maintain proper column orientation. Spin the column and collection tube at 750 g for 2 min. The purified sample will be collected in the bottom of the sample collection tube. Dry the sample in a vacuum centrifuge.

3. Preparation and Loading of the samples:

Resuspend the pellet in 4µl of the following reagent mixture containing 5µl demonised formamide and 1µl 25 mM EDTA with blue dextran (50 mg/ml). Centrifuge the solution to collect all the liquid at the bottom of the tube. Denature the samples at 95°C for 2 min and transfer them immediately on ice. The samples are then separated on polyacrylamide gel on the ABI PRISM 373 DNA Sequencer with the appropriate run module, DT {dR Set Any-Primer} mobility file, and matrix file.

DNA sequencing was done by R. Krug (MSZ, Würzburg).

V-2.3. Working with proteins

V-2.3.1. Immunoprecipitation

For immunoprecipitation of cellular proteins, protein G sepharose is used for monoclonal antibodies and goat antisera and protein A sepharose for rabbit antibodies. Incubate 20µl sepharose was with 0.5-4µg of antibody, 500-2000µg lysate and 300µl IP buffer. Depending on the actual experiment, lysates can be additionally precleared with beads if background problems arose. Samples are incubated on a rotator for at least 2 hr at 4 ^{o}C and then washed with different wash buffers, depending on the stringency required. The choice of wash buffer should insure low background and maximum preservation of complexed proteins. For example, when analysing a previously known strong interaction between two proteins, a strong wash buffer like RIPA is used to reduce background. When analysing novel interactions on the other hand, a mild wash buffer like TLB Buffer is used in order to insure that interactions between proteins of interest are not destroyed. However, the latter case may result in a higher background than using

RIPA Buffer. The immunoprecipitated proteins are boiled in Laemmli buffer and subjected to SDS PAGE and Western blotting, or *in vitro* kinase assay.

V-2.3.2. *In vitro* kinase assay

The kinase activity can be measured *in vitro* by radioactivity of [γ-^{32}P] ATP labelled substrate of the kinase. Wash the immunoprecipitated kinase twice both with TLB Buffer and Kinase Buffer. Add 5µCi [γ-^{32}P] ATP, 2-5µg of substrates, 0.1 mM ATP, and 20µl Kinase Buffer, incubate the mixture at 30°C for 20 min. The reaction is then stopped by adding SDS-loading Buffer and incubated at 95°C for 5 min. The proteins are separated by SDS-PAGE, and then blotted onto nitro-cellulose membranes (Diagonal). The membrane is either autoradiographically detected by a Phosphoimager. Equal loading is controlled by immunoblotting with specific antibodies. For the kinase assay with Myc-MyoD or GAL4-MEF2C: 500µg of the precleared cell lysate was immunoprecipitated with corresponding antibodies and used as substrate for ERK5WT or ERK5AEF. Kinase reactions are performed using double amounts of ATP, [γ-^{32}P] ATP, and Kinase Buffer.

V-2.3.3. Measurement of Protein concentration (Bio-Rad protein assay)

The Bio-Rad Protein Assay is based on the observation that when Coomassie Brilliant Blue G-250 binds to the protein the absorbency maximum shifts from 450 nm to 595 nm [229]. Add cell lysate containing 1-20µg of protein in to diluted Dye Reagent (1:5 dilution of Dye Reagent Concentrate in ddH_2O). Mix well and after a period of 5 min to 1 hr, measure the absorption at wavelength 595 versus reagent blank (containing the lysis buffer only).

V-2.3.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

Proteins can be separated largely on the basis of mass by electrophoresis in a polyacrylamide gel under denaturing conditions.

Collect transfected cells from each well (6 well plate) and wash with chilled phosphate-buffered saline (PBS). Lyse the cells in 50µl of Lysis Buffer. Cleared the crude cell lysates by centrifugation. Use 5µl 5x SDS-loading Buffer containing SDS to denature 20µl of precleared cell extracts at 95°C for 5 min. SDS is an anionic detergent that disrupts nearly all noncovalent interactions in native proteins. Mercaptoethanol is also included in the sample buffer to reduce disulphide bonds. The SDS complexes with the denatured proteins are then electrophoresed on a polyacrylamide gel in the form of a thin vertical slab. Vertical gels are set in between 2 glass plates with an internal thickness of 1.5 mm between the two plates. In this chamber, the acrylamide mix is poured and left to polymerise for at least 30 min at RT. The gels are composed of two layers: a 6-15% separating gel (pH 8.8) that separates the proteins according to size; and a lower percentage (5%) stacking gel (pH 6.8) that insures the proteins simultaneous entry into the separating gel at the same height.

	Separating gel	**Stacking gel**
Tris pH 8.8	2.5 ml.	1.25 ml
Acrylamide/bisacrylamide 29:1 (30%)	2.0-5.0 ml	1.7 ml
10% SDS	0.1 ml	0.1 ml
ddH_2O	5.4-2.4 ml	6.8 ml
10% APS	0.1 ml	0.1 ml

Pour the separating gel in between the two glass plates, leave a space about 1cm plus the length of the teeth of the comb. Add isopropanol to the surface of the gel. After the separating gel is polymerised, remove the isopropanol. Pour the stacking gel on top of the separating gel, insert comb, and let the gel polymerise. Load the samples in the wells of the slab and add running buffer in the chamber. A cover is then placed over the gel chamber and 200 volts are applied.

The negatively charged SDS-proteins complexes migrate in the direction of the anode at the bottom of the gel. Small proteins move rapidly through the gel, whereas large ones stay at the top. Proteins that differ in mass by about 2% can be distinguished with this method. The electrophoretic mobility of many proteins in SDS-polyacrylamide gels is proportional to the logarithm of their mass.

V-2.3.5. Immunoblotting

After the cell extracts subjected to SDS-PAGE, the proteins are transferred by electroblotting to nitro-cellulose BAS-85 membrane. Set up the apparatus (Bio-Rad) and blot at 400 mA in Blotting Buffer for 45 min. Use Ponceau S fixative dye solution (containing Ponceau S, trichloroacetic acid, and sulphosalicylic acid) to check if the transfer has been done. Stain for 5 min and wash with de-ionised water. For Western blot analysis, incubate the membranes in blocking buffer for 1 hr at RT or overnight at 4°C on a shaker. Dilute the first antibody in TBST, add to the membrane, and incubate at RT for 1 hr. Wash the membrane three times with TBST, each time for 10 min. Dilute Protein A-peroxidase (1:3000) in TBST as the secondary antibody, add to the membrane, incubate at RT for 45 min, and wash. This step is followed by the standard enhanced chemiluminescence reaction (ECL-system): incubate the membrane in a 1:1 mix of ECL solutions 1 and 2. This reaction is based on a peroxidase catalysed oxidation of Luminol, which leads to the emission of light photons, which can be detected, on X-ray film. Thus locating the peroxidase conjugated secondary antibodies bound to the primary antibody and the protein of interest.

V-2.4. Working with cell cultures

V-2.4.1. Maintenance of cell culture

C_2C_{12} mouse myoblast cell line, 10T1/2 mouse fibroblast cell line, and Phoenix-Eco (293T based), and Phoenix-GP+Eco packaging cell lines are maintained in DMEM (Life Technologies, Inc.) supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2 mM L-glutamine, and streptomycin/penicillin. The cells are cultured routinely to a 70% confluence and than splited.

To freeze cells, centrifuge cell cultures at 1100 rpm for 5 min and resuspend into freeze medium (70% DMEM supplemented with 10% FBS, P/S; 20% FBS and 1% DMSO) and aliquot them into cryotubes. These are set into a styropore box and left to freeze gradually in the -80°C freezer. The DMSO prevents ice crystal formation and allows the cells to remain intact. After 24 hr or later, transfer the cells to liquid nitrogen where they are kept for a longer period of time.

V-2.4.2. Calcium phosphate transient transfection assay

To a 15 ml tube add (for 10 cm plate):

- 5-10 µg DNA
- H_2O up to 450 µl
- 50 µl 2.5M$CaCl_2$
- 500 µl 2xBBS (pH 6.97) - add BBS to the mix on drops while vortexing

Leave mix for 20 min at room temperature.

The mix should appear turbid

Drop the mix to the cells to be transfected

Observe under the microscope small evenly distributed black DNA particles

Put the plates at 37°C under 7% CO_2 in the incubator and leave for 6 hrs to overnight

On the next day wash the cells with 1XPBS and stimulate them.

V-2.4.3. Lipofectamine 2000^{TM} transient transfection assay

1. The day before transfection, trypsinize and count the cells, plating them at 1-3X10^5 cells per well so that they are 90-95% confluent on the day of transfection. Cells are plated in 0.5 ml of their normal growth medium containing serum, if cells are normally cultured in serum, and without antibiotics.

2. For each well of cells to be transfected, dilute 0.8 to 1.0 µg of DNA into 50 µl of DMEM without antibiotics.

3. For each well of cells, dilute 1-3 µl of LF2000 Reagent into 50 µl medium (as ndicated above) and incubate for 5 min at room temperature.

4. Combine the diluted DNA (from step 2) with the diluted LF2000 Reagent (from step 3). Incubate at room temperature for 20 min to allow DNA-LF2000 Reagent complexes to form.

5. Add the DNA-LF2000 Reagent complexes (100 µl) directly to each well and mix gently by rocking the plate back and forth.

6. Incubate the cells at 37°C in a CO_2 incubator for a total of 24-48 h until they are ready to assay for transgene expression. It is not necessary to remove the complexes or change the medium. Alternatively, growth medium may be replaced after 4-6h without loss in transfection activity.

V-2.4.4. Luciferase reporter gene assay

Bioluminescence is characterized by light emmission produced in enzyme, such as firefly luciferase, which is assayed by measurement of light production upon addition of luciferin and ATP. The photon production by catalytic oxidation of beetle luciferin occurs from a enzyme intermediate, luciferyl-AMP.

Luciferin + ATP → luciferyl-AMP +PP

Luciferyl-AMP + O_2 → oxyluciferin + light

Luciferin is first activated by means of ATP. The activated luciferin reacts with oxygen to form dioxetane. Dioxetane deposes and excites the molecule, which transfers to its ground state by emission of fluorescence light.

Harvest cells from each well in 100µl of Lysis Buffer, mix well, and incubate on ice for 30 min. Precleare the crude cell lysates by centrifugation at maximum speed. 50µl of precleared cell extracts is added to 50 µl of Luciferase Assay Buffer. The activity is measured after injection of 50 µl of D-luciferin Solution, and the reaction is monitored in a Berthold luminometer for 5 sec. Normalise the luciferase activites on the β-galactosidase activity of co-transfected 1µg Rous sarcoma virus LTR β-gal vector in β-galactosidase assay. Results are presented as luciferase units normalised to protein concentration. Each experiment was done in duplicates or triplicates. The mean and standard deviations of at least three independent experiments are shown in the figures.

V-2.4.5. b-galactosidase assay

The β-galactosidase assay is performed according to a standard protocol (Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989: Molecular cloning: A laboratory Manual 2nd Ed., Cold Spring Harbor Laboratory Press, New York). 20µl (approximately 15-20µg of total protein concentration) of precleared cell lysate is added to 500µl of β-gal Assay Buffer and 100µl ONPG-solution. Incubate at 37°C till the solution turns yellow (between 4 hr to overnight). Add 250µl of 1 M Na_2CO_3 to stop the reaction. Measure the absorption of the solution at wavelength 420 within the next 30 min.

V-2.5. Retroviral infections

V-2.5.1. Transient transfection for production of infectious retroviruses

Production of infective retroviral particles and retroviral infections were done according to Pear et al. [230].

day 0

18-24 hours prior to transfection Phoenix cells are plated at density 1.5-2x10^6 per 10 cm plate in 10% FBS DMEM supplemented with 1% Penicillin-Streptomycin, 1% Glutamine.

day 1

About 5 min prior transfection, chloroquine was added to each plate at final concentration 25 µM. Cells were transfected using calcium phosphate method with 10 µg of different retroviral plasmid DNA and incubated for overnight at 37°C 7% CO_2.

day 2

Transfected cells were washed once with PBS and refed with 5 ml 10% DMEM and again left for overnight to produce viral particles. Successful transfection was determined visually for GFP expression.

V-2.5.2. Production of stably transduced C_2C_{12} cell lines using retroviral infections

day 3

Supernatant from transfected Phoenix cell lines was pipetted in 15 ml falcon tubes and centrifuged at 1100 rpm for 5 min to pellet cell debris and than filtered through 0,45µ filter. 700 µl of it was used to infect C_2C_{12} cells plated in 6 well plates at density 30 000 one day prior infection. To each well was added 4 mg/ ml polybrene. The medium was adjusted to 1 ml with 10% FBS DMEM and placed in the incubator. During the first 3 hours of infection cells were gently shaken every 15 min, and afterwards left for overnight.

The rest of the viral containing supernatant was frozen at -70°C.

day 4

C_2C_{12} cells were washed from the remaining virus and infected once again as indicated at day 3, this time using 1 ml of frozen viral supernatant. Cells were occasionally agitated during the first 3 hours and than incubated for additional 24 hours at 37°C.

day 5

Cells were washed once with PBS and selected for resistance to zeocin at concentration 400 μg/ml.

Successful infectants were selected depending on resistance to zeocin and expression of EGFP expressed from the same polycistronic mRNA as the gene of interest.

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VII. Appendix

AA	Amino acid
Ala	Alanine
Amp.	Ampicillin
APS	Ammoniumpersulphate
ATP	Adenosintriphosphate
bHLH	Basic helix-loop-helix
bp	Base pairs
C	Concentration
ca.	circa
cAMP	Cyclic adenosinmonophosphate
CDS	Coding sequence
cfu	Colony forming unit
CIP	Calf intestinal phosphatase
CKII	Casein kinase II
CR	Conserved region
CREB	cAMP-response element-binding protein
DAG	Diacylglycerol
DB	DNA-binding domain
DCM	Dichlormethane
(d)dNTP	(Di)Desoxynukleotidtriphosphate
DIC	Diisopropylcarbodiimid
DM	Diferentiation medium
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
D. Melanogaster	Drosophila melanogaster
DTT	Dithiothreitol
E. coli	Escherichia coli
ECL	Enhanced chemoluminiscence
EDTA	Ethylendiamintetraacetate
e.g.	for example (exempli gratia)
EGF	Epidermal growth factor
ERK	Extracellular signal regulated kinase
FBS	Fetal bovine serum
GDP	Guanosindiphosphate
GM	Growth medium
GTP	Guanosintriphosphate
HA	Hemagglutinine
His	Histidine
HLH	Helix-loop-helix
ID	Inhibitor of differentiation/DNA-binding
IL	Interleukin
IP	Immunprecipitation
IP_3	Inositol-1,4,5-triphosphate
JIP	JNK interacting protein
JNK	c-Jun N-terminal kinase
kb	kilobases
kbp	kilobasepairs
kDa	kilo-Dalton
Leu	Leucine
LiAc	Lithiumacetate

MAPK	Mitogen activated protein kinase
MAPKAP-K	MAPK activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MCK	Muscle creatin-kinase
MEF	Myocyte enhancer factor
MEK	MAPK/ERK activated kinase
MEKK	MAPK/ERK activated kinase Kinase
MKK	MAP kinase kinase
MLC	Myosin light chain
MNK	MAPK-interacting kinase
MSK1	Mitogen- and stress-activated protein kinase-1
MSZ	Institut für Medizinische Strahlenkunde und Zellforschung
MyoD	Myogenic determination gene
OD	Optical density
PAGE	Polyacrylamide-gelelektrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIP_2	Phosphatidylinsoitol-4,5-bisphosphat
PKC	Proteinkinase C
PLC	Phospholipase C
rpm	rounds per minute
RSK	Ribosomal S6 kinase
RTK	Receptor tyrosine kinase
SAPK	Stress-activated protein kinase
Ser	Serine
SH	Src homology
SLS	Sodium-laurylsarcosine
Sos	Son of sevenless
TAD	Transactivation domain
Trp	Tryptophane
Tyr	Tyrosine
V	Volt
v/Vol	Volume
w	Weight

Curriculum Vitae

Personal details

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Education

PhD in Molecular Biology

June 1997-

At the Institute for Medical Radiation and Cell Research, Julius-Maximilian University Würzburg, Germany, *Thesis title*: Analysis of the role of Extracellular Signal Regulated Kinase 5 (ERK5) in the differentiation of muscle cells

MSc in Biochemistry and Microbiology

September 1990-June 1995

At Sofia University St. Kliment Ohridsky, Sofia, Bulgaria

Thesis title: Effect of preparation isolated from cell walls of *Nocardia opaca* on the system of the complement and *Klebsiella pneumoniae* infection in mice

September 1977- May 1988

High School, Targovishte, Bulgaria

Languages

English (fluent); German (fluent – intermediary level course at University of Würzburg); Russian (fluent)

Publications	*Extracellular signal regulated kinase 5 (ERK5) is required for the differentiation of muscle cells*, **Dinev, D.**, Jordan, B.W.M., Neufeld, B., Lee, J.-D., Lindemann, D., Rapp, U.R., Ludwig, S., *EMBO* Reports, 2001, **2**(9) *Paper in press.*
	Serine/ Threonine kinase 3pK and MAPK-activated protein kinase 2 interact with the basic Helix-Loop-Helix transcription factor E47 and repress its transcriptional activity, Neufeld, B., Grosse-Wilde, A., Hoffmeyer, A., Jordan, B.W.M., Chen, P., **Dinev, D.**, Ludwig, S., Rapp, U.R., J. Biol. Chem., 2000, **275**(27) p. 20239:20242.
Memberships	Member of the Society of Signal Transduction (GST), Germany
Scientific Activities	Berlin, November 2000, Oral presentation at Millennium meeting of Signal Transduction Society (GST): “ERK5 is required the differentiation of muscle cells”.
Military Service	1988-1990