The Double-stranded RNA-binding Protein Staufen1 Negatively Regulates Skeletal Muscle Differentiation

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Abstract

Staufen1 is a double-stranded RNA-binding protein known to be involved in the transport, localization, decay and increased translation of some mRNAs. The goal of the present study is to determine the role of Staufen1 during myogenic differentiation by characterizing the effects of Staufen1 over-expression in C_2C_{12} cells. Immunofluorescence experiments revealed that Staufen1 over-expression causes a decrease in the fusion and differentiation indices and leads to the formation of myotubes with significantly fewer nuclei. We show, by western blot and qRT-PCR, that the protein expression of MyoD, myogenin and MyHC and the mRNA expression of MyoD, myogenin, Mef2A, Mef2C and p35 are significantly decreased during differentiation when Staufen1 is over-expressed. We then found that c-myc protein expression was increased during proliferation but that its mRNA expression remained unchanged. In this study we propose that Staufen1 negatively regulates skeletal muscle differentiation through the posttranscriptional regulation of c-myc, Mef2A, Mef2C and p35 transcripts.

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List of Abbreviations

AA: Amino acid

bHLH: Basic Helix-Loop-Helix

BrdU: 5-Bromo- 2’-deoxy-uridine

BSA: Bovine serum albumin

CAT: Chloramphenicol acetyltransferase

CDK: Cyclin dependent kinase

cDNA: Complementary deoxyribonucleic acid

CO_2 : Carbon dioxide

CUGBP1: CUG Triplet Repeat-binding Protein

DM: Differentiation media

DM1: Myotonic Distrophy type 1

DNA: Deoxyribonucleic acid

dNTP: Deoxynucleotide triphosphate

dsRBD: Double-stranded RNA-binding domain

EEJ: Exon-exon junction

GM: Growth media

GMC: Ganglion mother cell

HCl: Hydrogen chloride

HLH: Helix-Loop-Helix

HRP: Horseradish peroxidase

IGF: Insulin-like growth factor

IgG: Immunoglobulin G

MAPK: Mitogen-activated protein kinase

MCK: Muscle creatine kinase

MRF: Myogenic regulatory factor

mRNA: messenger Ribonucleic acid

MyHC: Myosin heavy chain

NaCl: Sodium chloride

NCBI: National Center for Biotechnology Information

PBS: Phosphate buffered saline

PBST: PBS with 0.05% tween-20

PCR: Polymerase chain reaction

PVDF: Polyvinylidene fluoride

qPCR: Quantitative Polymerase Chain Reaction

qRT-PCR : Quantitative Reverse Transcription Polymerase Chain Reaction

Rb: Retinoblastoma

RNA: Ribonucleic acid

RNP: Ribonucleoprotein complex

SBS: Staufen binding site

SDS: Sodium dodecyl sulfate

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TBD: Tubulin-binding domain

UTR: Untranslated Region

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1. Introduction

1.1. Skeletal muscle and myogenesis

1.1.1. Skeletal muscle

In mammals, there are three types of muscle tissue that can be identified based on their different contractile properties, structures and control mechanisms. One of these types of muscle tissue is skeletal muscle, which is made up multinucleated muscle fibers, or myotubes, bound together by connective tissue. The process by which these muscle fibers are formed is called myogenesis or muscle differentiation. Myogenesis is a highly complex and dynamic process which, in C_2C_{12} cells, begins with the generation of proliferating mononucleated myoblasts. Once differentiation is activated, these myoblast withdraw from the cell cycle and fuse together to form multinucleated mature myotubes. For over half a century, myogenesis has been studied extensively, leading to the discovery that a specific sequence of gene expression was shown to drive this process and to be necessary for proper myotube formation, both *in vivo* and *in vitro*.

1.1.2. Skeletal muscle differentiation

1.1.2.1. Primary regulators of myogenesis

Myogenesis is a complex process which is governed by the expression of members of the MyoD family of myogenic regulatory factors (MRFs) and Mef2 family of MADS-box myocyte enhancer-binding factors (Molkentin and Olson, 1996, Chang 2007). The ectopic expression of any of the MRFs in non-myogenic cell lines was shown to induce the expression of muscle specific gene (Hopwood et al., 1990, Dedieu et al., 2002). The ectopic expression of members of the Mef2 family of proteins, on the other hand, cannot initiate on its own the myogenic program

but cooperates with myogenic bHLH proteins to increase the transcription of myogenic genes (Molkentin et al., 1995).

These MRFs include MyoD, Myf5, MRF4 and myogenin (Dedieu et al., 2002), which possess a conserved DNA-binding domain and share about 80% protein sequence identity within a 70 amino acid (AA) sequence containing a basic helix-loop-helix (bHLH) domain (Edmondson et al., 1993). In MRFs, the bHLH has been shown to mediate heterodimerization with ubiquitous bHLH proteins known as E-proteins, such as E2A, E12 and E47. The myogenic bHLH protein/E-protein heterodimer then recognizes and binds a conserved consensus CANNTG (N standing for any base) sequence called an E-box, in the promoter region of muscle specific genes (Ferri et al., 2009). The binding of the heterodimer to this sequence activates transcription which is required for myogenesis to occur (Davis et al., 1990, Neuhold et al., 1993).

The MEF2 family, on the other hand, is made up of Mef2A, Mef2B, Mef2C and Mef2D (Molkentin et al., 1995) which share more than 85% sequence identity in a conserved 56 AA sequence called a MADS domain and an adjacent 27 AA region called the Mef2 domain. Unlike Mef2A, Mef2B and Mef2C which are ubiquitously expressed Mef2C expression was shown to be mostly restricted to muscle, brain and spleen (Martin et al., 1993, McDermotte et al., 1993). Mef2 factors have been shown to form homodimers or heterodimers that bind an A+T rich sequence (TAT/AAT/ATNGAC) found in a number of control elements of muscle specific genes in order to increase their transcription (Duprey et al., 1994, Dodou et al., 2003). As well as directly increasing gene transcription, Mef2 proteins also act as coregulators to increase the myogenic activity of MRFs, by binding to the myogenic bHLH protein/E-protein heterodimer and enabling the transcriptional activation of muscle-specific genes that lack E-boxes (Molkentin et al., 1995). It was also discovered that one particular member of the Mef2 family, Mef2C, was

also involved in a positive feedback loop, in which the C-terminus of myogenin increases Mef2C transcription. This feedback loop amplifies the myogenic signal by further increasing the expression of myogenic genes, including myogenin (Rogerson et al., 2002).

It is important to note that the genes activated during muscle differentiation do not necessarily all have either E-boxes or Mef2 biding sites in their promoter regions. It is also possible for some of those genes to be activated by both MyoD and Mef2 independently, through binding to their respective sequences. One such gene is myogenin, which possesses a Mef2 binding site as well as E-box sequences in its promoter region, meaning that both myogenic bHLH proteins and Mef2 proteins can activate its transcription (Ridgeway et al., 2000).

1.1.2.2. Activation of the myogenic program

In proliferating myoblasts that are already committed to the myogenic program, MyoD and Myf5 are expressed (Figure 1). In the presence of growth factors multiple proteins, including Id and c-myc and members of the E2F family of transcription factors, are responsible for maintaining proliferation and inhibiting differentiation.

Members of the Id family of proteins are dominant negative HLH proteins that are expressed at high levels during proliferation. Due to their lack of functional DNA binding domain (Arnold and Winter, 1998), they prevent the transcription activation of myogenic genes by binding to E-proteins and MyoD, sequestering them and inhibiting the formation of the MyoD/E2A heterodimer (Benezra et al., 1990, Jen et al., 1992). c-myc, on the other hand, was shown to inhibit differentiation by binding to a c-myc responsive region in the core promoter of the cyclin dependent kinase (CDK) inhibitor p21, inhibiting it transcription (Dang, 1999, Wu et al., 2003).

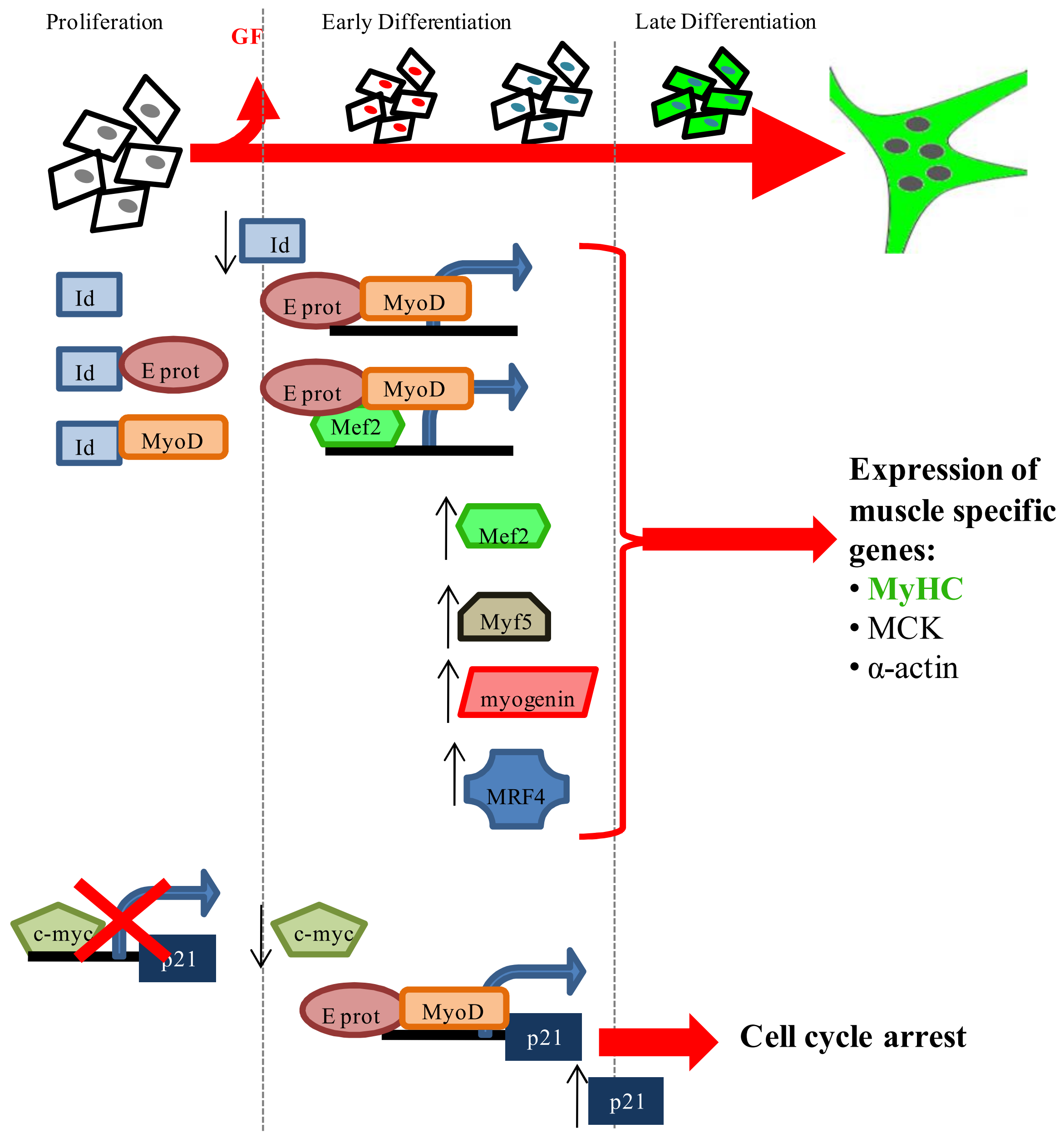


Figure 1: **Skeletal muscle differentiation.**

Skeletal muscle differentiation is a process in which proliferating mononucleated myogenic precursor cells, called myoblasts, fuse to become mature multinucleated myotubes. This process is tightly regulated and requires the expression of members of the family of myogenic regulatory factors and members of the family of Mef2 MADS-box myocyte enhancer binding factors. During proliferation, the expression of Id and c-myc prevents differentiation by inhibiting MyoD/E-protein transcription activity and preventing p21 expression. Once differentiation is induced by serum deprivation, Id and c-myc expression decreases, this increases MyoD/E-protein transcription activity and p21 expression. This increases the expression of myogenic genes and induces cell cycle exit, which ultimately leads to MyHC, MCK and α-actin expression and myoblasts fusion to form mature myotubes.

Inhibition of p21 expression leads to an increase in cyclin/CDK activity, which promotes cell cycle progression (Gartel andRadhakrishnan, 2005, Hermeking et al., 2000). Once differentiation is induced c-myc (Endo et al., 1986) and Id expression decreases (Benezra et al., 1990).

The decrease in Id proteins expression allows MyoD and E2A to form a heterodimer, which increases MyoD protein stability and initiates the differentiation program which leads to the increased expression of MyoD (Hatoum et al., 1998), Myf5 and Mef2 (Benezra et al., 1990). MyoD increases retinoblastoma (Rb) gene expression, which is known to promote myogenic gene expression (Novitch et al., 1999) via two mechanisms: by relieving MDM2-mediated repression of the ubiquitously expressed DNA-binding factor SP1 and increasing Mef2 transcriptional activity, in cooperation with MyoD (Grayson et al., 1998, Biesiada et al., 1999, Guo et al., 2003). MyoD and Mef2 then bind to the E-boxes and the Mef2 binding sites present in the control regions of myogenin and MRF4, activating their transcription and increasing their expression. The increase in myogenin expression increases the expression of other myogenic genes, including Mef2C, to further drive differentiation.

In order for the differentiation to occur, it not only requires the activation and expression of MRFs and Mef2 proteins but also requires myoblasts to undergo cells cycle withdrawal. During muscle differentiation, cell cycle exit has been shown to necessitate the increased translation and expression of both Rb and the CDK inhibitor p21. During differentiation Rb is hypophosphorylated and interacts with members for the E2F family of transcription factors which, as a result, represses the transcription of genes essential for cell cycle progression (Kitzmann and Fernandez, 2001). The expression of p21, on the other hand, is regulated by both MyoD and the proto-oncogene c-myc (Walsh and Perlman, 1997, Andrés and Walsh, 1996). Once differentiation is induced, c-myc protein levels decrease significantly, making it possible

for the MyoD/E-protein heterodimer to bind to the E-box in the promoter region of p21 and increase its transcription. The increased expression of p21 inhibits cyclin/CDK activity and ultimately leads to cell cycle arrest (Halevy et al., 1995).

Once the myoblasts have withdrawn from the cell cycle they then express muscle specific genes, such as myosin heavy chain (MyHC), muscle creatine kinase (MCK) and α-actin, and fuse to form mature multinucleated myotubes.

1.1.3. Pathways activating muscle differentiation

Two major signaling pathways have been shown to be responsible for the activation of the myogenic program: the p38 Mitogen-activated protein kinase (MAPK) pathway and the PI3K-AKT-p70S6K pathway.

1.1.3.1. p38 MAPK pathway

In mammals there are four p38 MAPKs which are phosphorylated by the MAPK kinases MKK6 and MKK3: p38α, p38β, p38γ and p38δ (Lluís et al., 2006). Through the use of specific inhibitors and knockdown experiments, it was determined that p38α positively regulates myogenic differentiation (Cuenda et al., 1999) while p38γ, on the other hand, negatively regulates differentiation (Gillespie et al., 2009, Lovett et al., 2010). The process by which p38α/β MAPK pathway is activated remains unclear. However, it has recently been proposed that the cell surface receptor, Cdo, through the Cdo-Bnp-2-Cdc42 pathway, is responsible for the activation of this pathway during myogenic differentiation (Kang et al., 2008). The cascade that follows the activation of this pathway and the process by which it activates the myogenic

program, are not yet completely understood. It was discovered, however, that p38α phosphorylates the members for the Mef2 family of regulatory factors, Mef2A and Mef2C, increasing their transcriptional activity (Zhao et al., 1999, Yang et al., 1999). This would contribute to the synergy between Mef2 and MyoD which is necessary to drive the differentiation process. The p38α/β MAPK pathway has also been shown to play a role in myogenesis through the phosphorylation of the Ser140 of the E-protein, E47. Phosphorylation of E47 is required for the formation of the MyoD/E47 heterodimer which binds the E-box in the promoter region of muscle specific genes to activate their transcription (Lluís et al., 2005). Activation of the p38α/β MAPK pathway has also been shown to be essential for the recruitment of the chromatin remodeling complex to regulatory regions of myogenic genes. The recruitment of this complex then allows binding of the myogenic bHLH protein/E-protein heterodimers to the E-box sequence which otherwise would be inaccessible (Simone et al., 2004). It was also determined that the p38α/β MAPK pathway facilitates the recruitment of RNA polymerase II to muscle specific promoters, without which transcription could not occur (Lluís et al., 2006). Regardless of the fact that a specific sequence of event has not yet been identified to explain how this pathway activates the myogenic program, all of these findings point to the p38α/β MAPK pathway as being one of the main pathways required for the induction of muscle differentiation.

1.1.3.2. PI3K-AKT-p70S6K pathway

In response to an external stimulus, Insulin-like growth factor (IGF) signaling activates the PI3K-AKT-p70S6K pathway required for myogenesis (Kaliman et al., 1998). The specific cascade that makes up this pathway is complex (Figure 2). Nevertheless, key steps have been

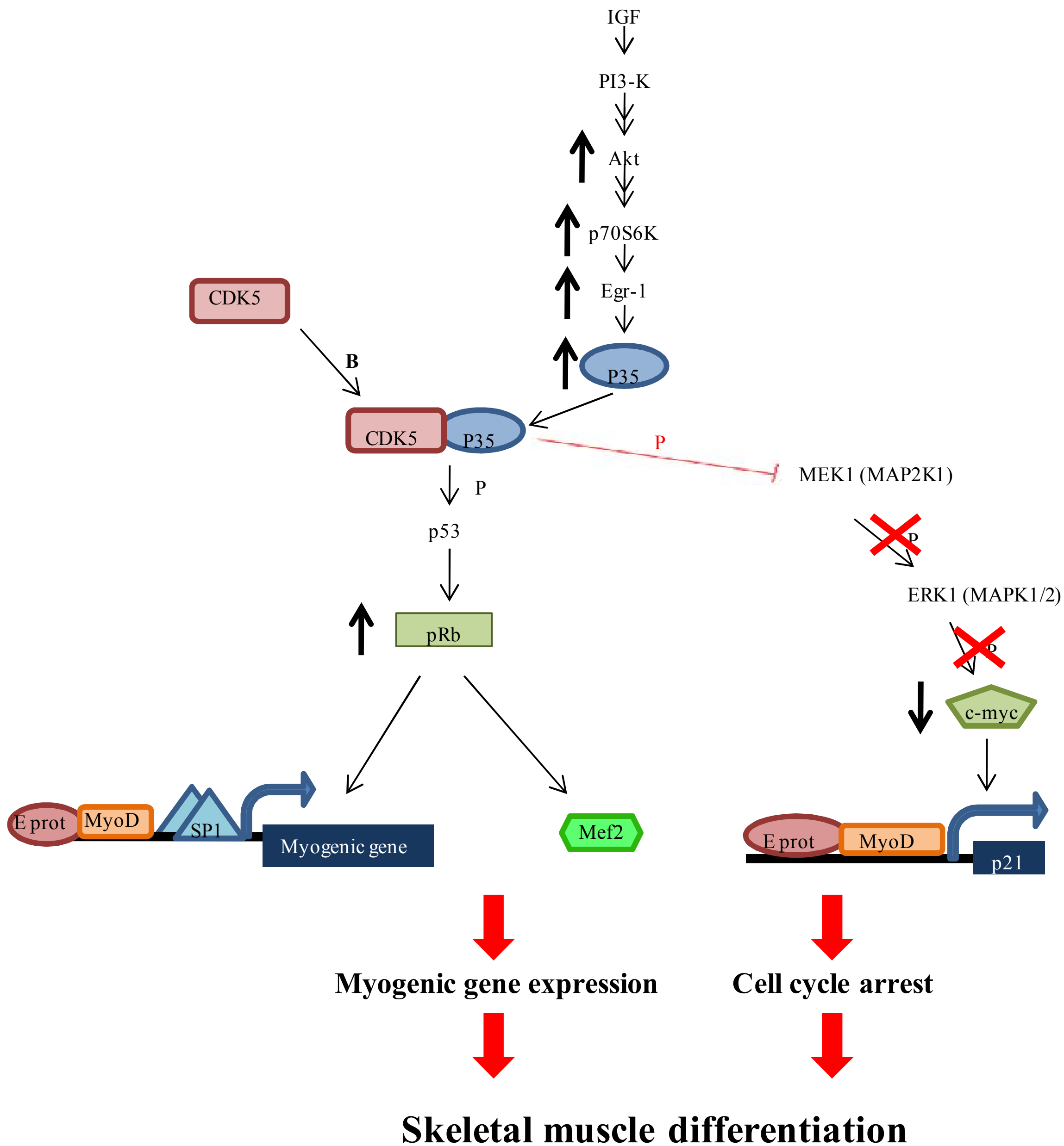


Figure 2: **Activation of the PI3K-AKT-p70S6K pathway during myogenesis.**

The PI3K-AKT-p70S6K pathway is required for skeletal myogenesis. Once differentiation is induced by serum deprivation, the IGF signaling pathway is activated, which activates PI3K, which increases the expression of AKT, p70S6K and Egr-1 sequentially. The increase in Egr-1 increases p35 transcription, which binds (B) CDK5 consequently increasing p35/CDK5 activity. Activation of p35/CDK5 phosphorylates (P) p53 and MEK1, which increases myogenic gene expression and inhibits the MEK/ERK pathway and in the end leads to cell cycle arrest and skeletal muscle differentiation.

identified. One of these steps is the increased expression of early growth response gene-1 (Egr-1) (Harada et al., 2001a), which increases p35 transcription (Harada et al., 2001b). The increased p35 expression, increases p35 binding to the cyclin-dependent kinase 5 (CDK5), which increases CDK5 activity (Lazaro et al., 1997, Amin et al., 2002, Sarker et al., 2004). Once activated, p35/CDK5 regulates two distinct pathways that promote differentiation. On one hand, p35/CDK5 drives myogenesis by phosphorylating and stabilizing p53, a protein shown to be required for myogenesis (Soddu et al, 1996, Tamir and Bengal, 1998). It has been proposed that p53 promotes myogenesis, by binding to a p53-binding site in the *Rb* promoter region and increasing its transcription (Porrello et al., 2000). On the other hand, p35/CDK5 promotes differentiation by phosphorylating MEK1 (MAP2K1), inhibiting the MEK1-ERK1/2 pathway (Sharma et al., 2002), which under normal circumstances was shown to phosphorylate Ser62 of c-myc increasing its stability (Sears et al., 2000). Activation of the PI3K-AKT-p70S6K pathway therefore induces skeletal muscle differentiation and fusion by increasing p35/CDK5 activation, which increases p53 and decreases c-myc protein stability.

1.2. The double-stranded RNA-binding protein: Staufen

1.2.1. Staufen in Drosophila

Staufen is a double-stranded RNA-binding protein that was first discovered in *Drosophila* where it was first shown to be required for the development of anterior-posterior polarity during oogenesis (Schupbach et al., 1986). Two key mRNAs are involved in determining polarity: *bicoid* and *nanos*. The first encodes the anterior signal and is localized to the anterior pole of the egg where it is then translated to form a bicoid gradient (St Johnston et al., 1991,). The second

forms a similar gradient in association with the *oskar* mRNA in order to determine the localization of the posterior pole of the egg. The transport and proper localization of both *bicoid* and *oskar* mRNAs was shown to be dependent on Staufen (Roegiers et al., 2000).

The role of Staufen in *Drosophila* is not limited to oogenesis. It has also been shown to participate in the embryonic development of the central nervous system, where it was shown to be involved in establishing asymmetric cell division: a process in which a cell divides to produce two daughter cells that have different cell fates. In this case neuroblasts delaminate from the epithelium and divide to generate as small ganglion mother cell (GMC) and another neuroblast (Campos-Ortega et al., 1997). This process requires the asymmetric distribution of *miranda* and *prospero*. Staufen has been shown to interact with the 3'UTR of *prospero* and be required for the proper basal localization of *prospero* mRNA and the *staufen/prospero/miranda* complex, which following mitosis is segregated to the GMC (Broadus et al., 1998)

1.2.2. Staufen in mammals

Two mammalian Staufen homologues sharing 51% AA sequence identity have been found: Staufen1 and Staufen2. In humans, Staufen1 is located on chromosome 20, position q13.1, and Staufen2 is located on chromosome 8, position q13-q21.1. The Staufen1 protein consists of four double-stranded RNA-binding domains (dsRBD), dsRBD2 to dsRBD5 and a tubulin-binding domain (TBD) in its C-terminus. Staufen2 protein, on the other hand, has dsRBD2 to dsRBD4, an additional dsRBD that Staufen1 does not possess and a C-terminal microtubule-associated domain (Figure 3). The dsRBD3 and dsRBD4 were shown to interact with the phosphate backbone of the double-stranded RNA, while dsRBD2 and dsRBD5 are truncated and are unable

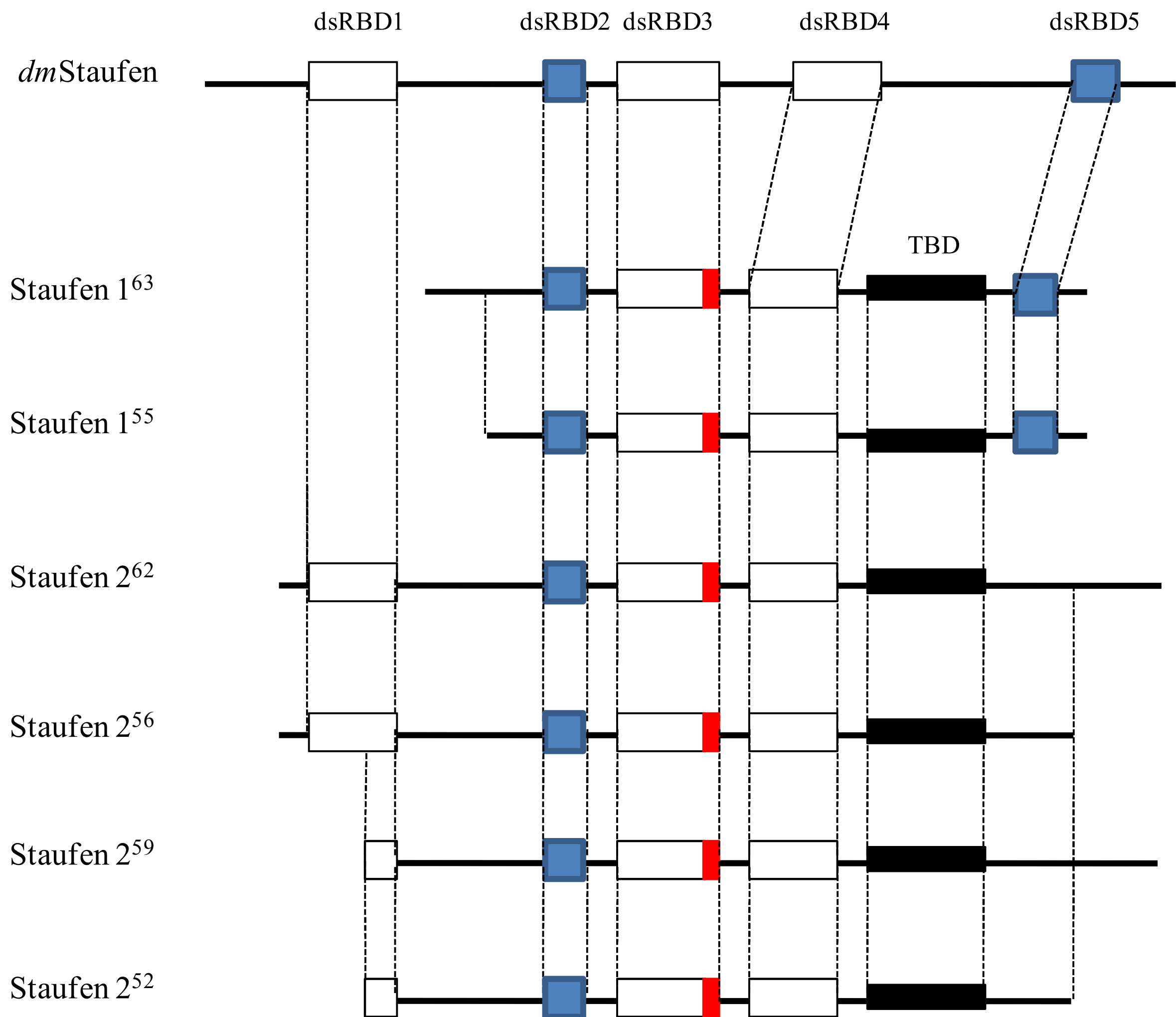


Figure 3: **Schematic representation of the different domains of the hStau1 and hStau2.**

The RNA-binding protein Staufen was first discovered in *Drosophila*. *Drosophila* Staufen (*dm*Staufen) possess five double-stranded RNA-binding domains (dsRBD): three of which are functional, dsRBD1, dsRBD3 and dsRBD4, and two others that are not, dsRBD2 and dsRBD5. Two mammalian homologues sharing 51% AA sequence identity were later discovered: Staufen1 and Staufen2. Both of which can be found in different splicing isoforms: Staufen1^{63}, Staufen1^{55}, Staufen2^{62}, Staufen2^{59}, Staufen2^{56} and Staufen2^{52}. Staufen1 possesses a tubulin binding domain (TBD) and dsRBD2 to dsRBD5, while Staufen2 possesses TBD and dsRBD1 to dsRBD4. Both Staufen proteins were also shown to possess a nuclear localization signal (Red) in the dsRBD3, enabling them to shuttle in and out of the cytoplasm.

to bind RNA (Martel et al., 2010). Both genes can be found in different splicing isoforms: Staufen1^{63} (63 kDa), Staufen1^{55} (55kDa) and Staufen1^{i} (Duchaîne et al., 2002) for Staufen1 and Staufen2^{62} (62kDa), Staufen2^{59} (59kDa), Staufen2^{56} (56 kDa) and Staufen2^{52} (52 kDa) for Staufen2 (Duchaîne et al., 2000, Furic et al., 2008). However most of the work that has been done on Staufen1 and Staufen2 has been done with the Staufen1^{55} and Staufen2^{62} isoforms. In mammal both Staufen proteins are found primarily in the cytoplasm were they are thought to take part in mRNA localization (Kiebler et al., 1999).

1.2.2.1. Staufen2

Staufen2 is a protein whose expression was revealed to be important in neurons (Duchaîne et al., 2002), where it was shown to forms ribonuclear protein (RNP) complexes, which enabled dendritic mRNA transport, mediated by its C-terminal microtubule-associated domains through its association with a kinesin motor protein (Jeong et al., 2007). It was also found to contain a nuclear localization signal directly following the dsRBD3 giving it the ability to not only bind RNA with high affinity but to also shuttle in an out of the nucleus. In the nucleus, it has the ability to bind a specific subset of RNAs to form RNPs and transport them out into the cytoplasm, through either an exportin-5 (for the 62-kDa isoform) (Macchi et al., 2004) or CRM1 (for the shorter 59kDa isoform) dependent mechanism (Miki et al., 2004). The study of Staufen2 is not limited to its role in neurons; it has also been shown that Staufen2 is expressed in muscle, where it is thought to be an important component of the postsynaptic apparatus and be involved in the maturation and plasticity of the neuromuscular junction (Bélanger et al., 2003).

1.2.2.2. Staufen1: a multi-functional protein

1.2.2.2.1. Staufen1 and dendritic mRNA transport and localization

Unlike Staufen2, Staufen1 is ubiquitously expressed. It was shown, by double-immunofluorescence experiments, that Staufen1 colocalizes with the rough endoplasmic reticulum in mammalian (Luo et al., 2002) and human cells (Marión et al., 1999). It was also discovered, by sedimentation on sucrose gradient, that Staufen1 was associated with polysomes and more specifically with the ribosomal subunits 40S and 60S (Marión et al., 1999). Like Staufen2, Staufen1 was also shown to enable mRNA transport in association with tubulo-vascular structures, by binding a specific subset of mRNAs to form RNPs in dendrites (Furic et al., 2008). Unexpectedly, Staufen1 containing RNPs do not appear to colocalize with the Staufen2 containing RNPs suggesting that they possess different roles in neurons (Kiebler et al., 1999). It was later shown, however, by DNA microarray analysis with probes derived from mRNAs isolated from immunopurified Staufen1 and Staufen2 RNPs, that a very low percentage of mRNAs were associated with both Staufen1 and Staufen2 in RNPs (Furic et al., 2008). However, for the most part Staufen1-RNPs and Staufen2-RNPs are associated with unique but overlapping sets of mRNAs that are involved in cell metabolism, regulation of cellular activity, transcription, catalytic activity and transport (Furic et al., 2008). The formation of Staufen1 containing RNPs was found to be essential for synapse development which is necessary for proper locomotor activity. This was shown by homologous recombination of the mouse Staufen1 gene, which generated a truncated form of Staufen1 lacking a functional dsRBD3. Cultured hippocampal neurons derived from these mice exhibited a decrease in dendritic spine morphogenesis and failed proper dendritic Staufen1-RNP delivery (Vessey et al., 2008).

1.2.2.2.2. Staufen1 is capable of nuclear-cytoplasmic shuttling and is involved in stress granule formation

In addition to being able to transport RNA within the cytoplasm, it was also demonstrated, using a yeast import assay, that Staufen1 is able to shuttle from the cytoplasm into the nucleus, in a CRM1 (Miki et al., 2004) and exportin-5 (Macchi et al., 2004) independent manner, via the bipartite nuclear localization signal (NLS) at the C-terminal end of the dsRBD3, a domain that was also identified as being the primary RNA-binding domain (Martel et al., 2006). The role of Staufen1 in mammalian cells does not limit itself to mRNA transport. It has also been shown, by immunohistochemistry, to be present and involved in the formation of stress granules induced in different cell line, where it is thought to help recovery from stress by stabilizing polysomes (Thomas et al., 2005, Thomas et al., 2009).

1.2.2.2.3. Staufen1 enables CAP-dependent translation

Staufen1[55] was shown to be implicated in CAP-dependent translation of some mRNAs. This was determined by generating reporter constructs containing an HIV type1 *trans*-activating response (TAR) element or other RNA structures tethered to the 5'end of a reporter transcript. Using these constructs the authors found that Staufen1 increased reporter activity both in rabbit reticulocyte lysates and in human embryonic kidney 293T cells. However, they did not observe an increase in reporter activity in cases where an RNA structure was not present in the 5'end of the reporter transcript. From this, they concluded that Staufen1 stimulates translation of mRNAs that possess structured 5'UTR (Dugré-Brisson et al., 2005).

1.2.2.2.4. Staufen is involved in mRNA decay

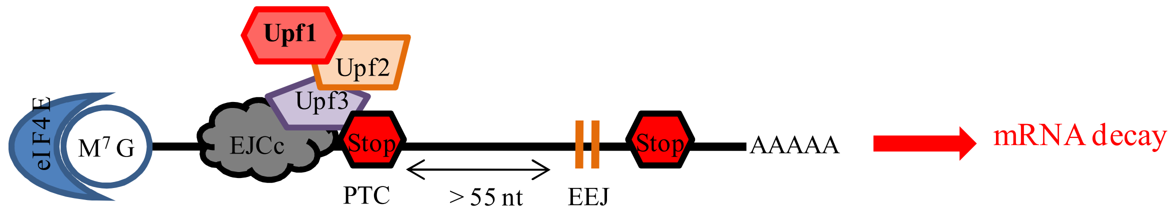
mRNA decay is an important cellular process that both regulates gene expression and protects the cells from deleterious effects of non-sense mutations. Two distinct decay mechanisms regulate this process: Nonsense-mediated decay (NMD) and Staufen-mediated decay (SMD)

NMD is a surveillance mechanism that degrades mRNAs that possess premature termination codons (PTC) situated more than 50 and 55 nucleotides upstream of an exon-exon junction (Figure 4). This mechanism prevents the translation of truncated proteins that could have deleterious effects on normal cell function. Other than degrading mRNA with PTCs, NMD serves a regulatory function by degrading specific transcripts to regulate their expression (Maquat and Gong, 2009). NMD occurs during pre-mRNA processing and splicing, by the formation and maintenance of an exon junction complex (EJC) upstream of an exon-exon junction. This process requires the sequential assembly of three up-frameshift proteins: Upf1, Upf2 and Upf3. During normal mRNA processing an EJC is formed which recruits Upf3 and Upf2. If translation terminates prematurely the EJC/Upf3/Upf2 complex remains bound to the transcript and recruits Upf1 through translation release factors or Upf2 to form an active NMD complex (Singh and Lykke-Andersen, 2003). Once Upf1 is recruited it triggers a 5’ and 3’ exonucleolytic RNA degradation which degrades the mRNA from both the 5’ and 3’ ends (Lejeune et al., 2003).

SMD, on the other hand, is a translation dependent mechanism that conditionally regulates the expression of normal transcript (Figure 4). The possibility that Staufen1 could be involved in an mRNA decay mechanism came from the results of a yeast two-hybrid analysis which

A

NMD



B

SMD

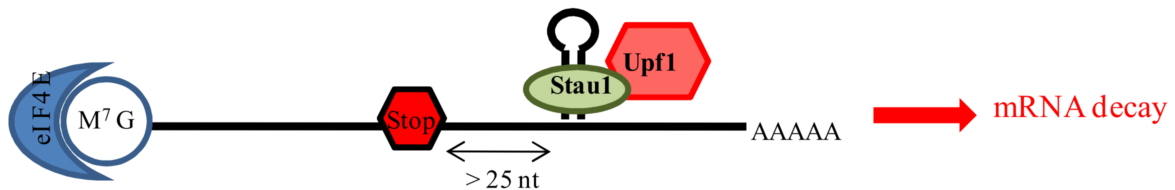


Figure 4: **mRNA decay mechanisms: NMD and SMD.**

(A) NMD is a surveillance mechanism which degrades mRNAs that possess premature termination codons (PTC) more than 55 nucleotides (nt) upstream of an exon-exon junction (EEJ), protecting the cell from deleterious effects of truncated proteins. During mRNA processing an exon junction complex (EJC) is formed which recruits Upf3 then Upf2. If translation ends prematurely, the EJC/Upf3/Upf2 complex remains bound and recruits Upf1 to form an active NMD complex which degrades the mRNA from both the 5' and 3' ends. (B) SMD is a mechanism that regulates the expression of normal transcripts that possess a hairpin structure called a Staufen binding site (SBS) in their 3'UTR. Staufen1 recognizes and binds the SBS of specific transcripts and recruits Upf1 to form an active SMD complex. Activation of this complex leads to the degradation of the SBS containing mRNA.

identified Staufen1 as being a protein that interacts with Upf1(Kim et al., 2005). It was later determined that unlike in NMD, transcripts that are degraded by SMD do not have to derive from intron containing genes, they do not need to undergo splicing and they do not require an EJC made up of Upf2 and Upf3. Nevertheless, SMD like NMD was shown to require binding with Upf1 in order to be activated, which theoretically would lead to possible competition between the two mechanisms for access to Upf1 (Gong et al., 2009). It has been determined that in order for SMD to occur, Staufen1 must recognize and bind a Staufen binding site (SBS) in the 3'UTR, 25 nucleotides downstream of a STOP codon, of transcripts that are actively being translated (Kim et al., 2005). Once bound, it recruits Upf1 which binds to the Staufen1 region containing the dsRBD4 and the TBD (Gong et al. 2009). This activates the SMD complex and leads to degradation of the mRNA.

1.2.3. Staufen1 in muscle and muscle differentiation

For more than two decades, most of the research done on Staufen has been concentrated on the role of both Staufen1 and Staufen2 in neurons. More recently however, the laboratory of Dr. Bernard J. Jasmin began looking at the potential role of Staufen in muscle. His group showed, by immunohistochemistry, that in skeletal muscle, Staufen1 and Staufen2 were found to be enriched in the postsynaptic sarcoplasm of muscle fibers and newly formed ectopic synapses, indicating that they are important components of the mammalian neuromuscular junction (Bélanger et al., 2003). Both Staufen homologues were also shown, by western blot, to be present, expressed primarily as the $Staufen1^{55}$ and $Staufen2^{59}$ isoforms, in slow as well as fast muscle, two types of muscle with different contractile properties. Following denervation *in vivo*

and treatment of myotubes with neurotophic factors that induce the formation of ectopic synapses such as agrin and neuregulin, Staufen1 and Staufen2 expression levels were shown to increase rapidly (Bélanger et al., 2003). They have also looked, by western blot, at the expression of endogenous Staufen1 during C_2C_{12} myoblast differentiation and found that its levels were increased in four day old myotube compared to myoblast (Bélanger et al.2003).

The laboratory of Dr.Lynne E. Maquat replicated the experiment described previously and also observed that endogenous Staufen1 levels were increased in myotubes compared to myoblast (Kim et al., 2007). This group, in collaboration with the group of Dr.Luc DesGroseillers, is responsible for the discovery that Staufen1 is involved in the decay of specific transcripts through SMD (Kim et al., 2005). They proposed that Staufen1, through SMD, positively regulated skeletal muscle differentiation by competing with NMD for access to Upf1 (Gong et al., 2009). They identified SMD targets that give rise to proteins that are involved in skeletal muscle differentiation by microarray analysis, which they then confirmed by RNA immunoprecipitation and RT-PCR. They found that SMD targets, which give rise to proteins known to inhibit myogenesis, decreased during differentiation, like c-jun and Pax3 (Gong et al., 2009), while those that were shown to be NMD targets, giving rise to proteins known to promote differentiation, such as myogenin increased during differentiation. Since binding to Upf1 is required in both NMD and SMD, they concluded that SMD competed with NMD to promote myogenesis.

The laboratory of Dr.Kenji Irie, on the other hand, found that Staufen1 negatively regulates myogenic differentiation and that its role in differentiation was not mediated by SMD (Yamaguchi et al., 2008). They knocked-down endogenous Staufen1 expression in C_2C_{12} cells and noticed that differentiation occurred spontaneously without induction by serum deprivation

and was accompanied by an increase in the expression of muscle-specific genes myoglobin and myogenin. In order to identify if SMD was involved in this process, they knocked down Upf1 by siRNA and observed that, unlike cells where Staufen1 was knocked down, these cells did not express myogenin without induction. They also transfected cells, in which endogenous Staufen1 was knocked down, with Staufen1[i], a Staufen isoforms with reduced RNA binding capacity, and did not observe any difference in myogenin protein expression compared to Staufen1 transfected cells. From this, they concluded that SMD was not involved in mediating the effects of Staufen1 in C_2C_{12} differentiation.

Even though two independent groups have endeavored to determine the role of Staufen1 in skeletal muscle differentiation the results remain inconclusive and controversial.

1.3. RNA-binding proteins and their roles in skeletal muscle differentiation

RNA-binding proteins have been shown to regulate gene expression, through the posttranscriptional regulation of some transcripts, during different cellular processes such as proliferation, differentiation and apoptosis (Glisovic et al., 2008). This process requires binding of an RNA-binding protein to a specific sequence of the transcript which, depending on the situation, then changes the stability of the transcript, increases translation or targets the transcripts for decay. Two examples of RNA-binding proteins that have been shown to be important in the posttranscriptional regulation of transcripts during skeletal muscle differentiation are HuR and CUGBP1.

HuR is an RNA-binding protein that binds a sequence, called an AU-rich element (ARE), in the 3'UTR of specific transcripts (Myer et al., 1997). Binding of HuR to an ARE has been shown

to have a protective effect on the transcript, by preventing its rapid degradation, which then increases its abundance (Peng et al., 1998). Knocking down HuR in C_2C_{12} completely inhibited skeletal muscle differentiation and led to a significant decrease in the expression of myogenic regulatory factors such as MyoD, myogenin and p21 (Van der Giessen et al., 2003). It was discovered that HuR, which normally is sequestered in the nucleus during proliferation, would shuttle out of the nucleus and into the cytoplasm at the onset of differentiation. Once in the cytoplasm, it binds the regulatory factors MyoD, myogenin and p21 transcripts and increase their stability and abundance which drives the differentiation process (Figueroa et al., 2003). HuR, therefore, plays a crucial role during skeletal muscle differentiation through the stabilization of transcripts whose increased expression is required for the activation of the myogenic program.

The RNA-binding protein CUGBP1 has been extensively studied in the neuromuscular disorder, Myotonic dystrophy type 1 (DM1), where it was shown to bind the CTG repeat expansion of the mutant DMPK gene, responsible for the pathology. In DM1 patients, it was discovered that CUGBP1 steady state levels and half-lives were increased (Kuyumcu-Martinez et al., 2007) and that this increase was responsible for a wide array of symptoms, including muscle wasting (Orengo et al., 2008) and impaired myogenic differentiation (Timchenko et al., 2001). During normal skeletal muscle differentiation, CUBP1 was shown to be upregulated in the cytoplasm, where it binds to a CAG and CUG repeat in the MyoD and p21 transcripts, respectively, and increase their translation (Timchenko et al., 2001, Timchenko et al., 2003). It was determined that in the DM1 pathology, CUGBP1 cytoplasmic abundance does not increase during skeletal muscle differentiation, which leads to a decreased expression of Mef2A and p21 and consequently impairs myogenesis (Timchenko et al., 2001). From this example we can conclude that CUGBP1 also plays a crucial role during skeletal muscle differentiation. However,

this time it is not through the stabilization of transcripts whose increased expression is required for the activation of the myogenic program, but rather through the increased translation of two proteins required for differentiation and cell cycle arrest.

These two examples demonstrate the importance of RNA-binding proteins, as mediators of posttranscriptional regulation, in skeletal muscle differentiation.

1.4. Rationale

During the last two decades, the RNA-binding protein Staufen1 was extensively studied in neurons, where it was shown to play an important role in mRNA transport and localization. Later work revealed that Staufen1 was also involved in the posttranscriptional regulation of transcripts, through increased translation and mRNA decay. Not much is known about Staufen1 and its role in muscle but research from our laboratory has shown that Staufen1 was expressed in skeletal muscle, where it is thought to be an important component of the postsynaptic apparatus at the neuromuscular junction.

Since Staufen1 is expressed in muscle and has been implicated in the posttranscriptional regulation of some transcripts, we would assume that, like the RNA-binding proteins HuR and CUGBP1, it too plays an important role in regulating skeletal muscle differentiation. As it was mentioned previously, two studies have already been done on the subject but have yielded opposite results (Kim et al., 2007, Yamaguchi et al., 2008, Gong et al., 2009). We therefore decided to take a new approach to determine the role of Staufen1 in skeletal myogenesis. We generated C_2C_{12} stable cells lines over-expressing Staufen1 and observed its effects on the differentiation process. The use of stable cell line ensures the fidelity and reproducibility of our

results, since we remove transfection efficiency as a possible variable. The results obtained from our stable cell lines, in combination with our knowledge of the literature, will enable us to infer a mechanism or mechanisms by which Staufen1 mediates its effect on muscle differentiation.

1.5. Hypothesis

The double-stranded RNA-binding protein Staufen1 is a negative regulator of skeletal muscle differentiation.

1.6. Objectives

i. Generate stable C_2C_{12} cell lines over-expressing mouse Staufen1.
ii. Observe the effects of Staufen1 over-expression on C_2C_{12} differentiation and fusion.
iii. Identify possible mRNA targets of Staufen1 that would mediate the effects observed on C_2C_{12} cell proliferation and differentiation when over-expressed.

2. Materials and Methods

2.1. Cell culture

C_2C_{12} myoblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) (Thermo Scientific HyClone) growth media (GM) supplemented with 10% fetal bovine serum (FBS, VWR), and 1% penicillin/streptomycin solution (GIBCO). Cells were incubated at 37°C in 5% CO_2. The cells were not permitted to reach 90% confluency unless differentiation was required. For experiments that require myogenic differentiation, cells were seeded at the same density on Matrigel (BD biosciences) coated 35 mm plates and cultured until they reached 90% confluency. Differentiation was then induced by replacing the GM with a low-serum differentiation media (DM) containing DMEM supplemented with 2% horse serum (GIBCO) and 1% penicillin/streptomycin solution (GIBCO). The DM was replaced every 48 hours for the duration of the time-course.

2.2. Transient transfection

C_2C_{12} cells were seeded at low density on 35mm plates and left to proliferate overnight in GM as described previously. The next morning, the cells were transfected with different constructs depending on the experiment (Table 1). For each 35mm well, 1µg of DNA was added to 100µL of OptiMEM (Gibco) and 6µL of Plus Reagents (Invitrogen) and incubated for 15 minutes at room temperature. After which, the first solution was added to a second solution containing 100µL of OptiMEM (Gibco) and 6µL of Lipofectamine Reagent (Invitrogen) and incubated a second time for 15 minutes at room temperature. During this time, the GM covering

the cells was replaced with DMEM. Afterwards, 200μL of the final solution was added to the DMEM and the cells were incubated at 37°C in 5% CO_2. After 5 hours of incubation the transfection media was aspirated, the cells were washed once with sterile PBS and GM was added. The cells were cultured as described previously until needed for an experiment.

2.3. Generation of stable cell lines over-expressing Staufen1

C_2C_{12} cells were cultured as described previously. Once they reached 70% confluency the cells were transfected with either a pcDNA-Staufen1-HA or an empty pcDNA3 vector, using Lipofectamine reagent (Invitrogen), as described previously. Once the transfections were complete the cells were cultured in GM. In order to obtain stable lines, the transfected cells were selected for several weeks using 1mg/mL of neomycin (G418) in GM. Once the selection process was complete the cells were seeded at very low density on 100mm plates. Clonal colonies were isolated and screened for the presence of the tagged protein by Western blotting using a mouse anti-HA primary antibody, as described later. Stocks of the positive clones were frozen and kept in liquid nitrogen. Selection was maintained by adding 500μg/mL of G418 to the GM.

2.4. Immunohistochemistry

Stable clones and a pcDNA3 control were seeded on Matrigel coated 35 mm plates and, depending on the experiment, differentiation was induced. During proliferation or four days after induction of differentiation the cells were rinsed with phosphate buffered saline (PBS), fixed

with 1% formaldehyde at room temperature for 10 minutes and then washed three subsequent times in PBS. Afterwards, the cells were incubated in permeabilizing buffer (0.5% Triton X-100 in PBS) for 10 minutes at room temperature and quickly rinsed with PBS to remove the excess. The cells were then incubated in blocking buffer, containing 1% bovine serum albumin (BSA) in PBS), at room temperature for 10 minutes. Next, they were incubated with a 1:1 dilution of MF20 hybridoma supernatant or a 1:2500 dilution of a mouse anti HA.11 primary antibody (Covance, 16B12) in 1% BSA-PBS for one hour at 37°C and washed three times with PBS. After that, cells were incubated one last time with a 1:2000 dilution of anti-mouse Alexa Fluor488 or anti-mouse Alexa Fluor594 secondary antibody (Invitrogen) in 1% BSA-PBS for one hour in the dark at 37°C. They were then washed three final times with PBS and cover slips were mounted with Vectashield mounting medium containing DAPI (Vector laboratories Inc.). Cells were observed through a Zeisse 2.5X or 10X objective, with a Zeiss Axioskop2 mot plus instrument. Images were captured through different fluorescent filters using a Qimaging Micropublisher digital imaging system and merged with the Northern Eclipse software.

2.5. Calculation of the FI, DI and the numbers of nuclei/myotube

To determine the fusion and differentiation potential of Staufen1 over-expressing C_2C_{12} cells and compare it to that of wild type C_2C_{12} cells, the Differentiation index (DI), Fusion index (FI) and the average number of nuclei per myotube of clones #15, #25 and the pcDNA3 control cells were calculated, using the pictures taken by fluorescence microscopy. The DI was calculated by counting the number of nuclei in MyHC positive cells and dividing it by the total number of nuclei. The FI was calculated by counting the number of nuclei in MyHC positive cells with

three or more nuclei (myotube) and dividing it by the total number of nuclei. Finally, average number of nuclei per myotube was calculated by counting the number of nuclei in MyHC positive cells with three or more nuclei and dividing it by the number of MyHC positive cells with three or more nuclei.

2.6. Quantitative RT-PCR

2.6.1. RNA extraction

Staufen1 over-expressing clones #15 and #25, as well as a pcDNA3 control cell line were seeded at the same density on Matrigel coated 35 mm plates or six well plates. Depending on the experiment differentiation was or was not induced. At specific times during proliferation or differentiation, the medium was aspirated and the cells were rinsed twice with cold sterile PBS. The cells were put on ice, 1mL of Trizol reagent (Invitrogen, Life Technologies) was added to each well and the cells were scraped using a cell scrapper. The samples were then transferred to individual 1.5mL microtubes and left to incubate for 5 minutes at room temperature. 200µL of chloroform was added to each sample, which were then shaken, left to incubate for 2 minutes at room temperature and then centrifuged at 12000g for 15 minutes at 4°C. Afterwards, the aqueous phase from each sample was transferred to a new 1.5mL microtube, where RNA was precipitated by adding 500µL isopropyl alcohol. The samples were then incubated overnight at -80°C, in order to increase the yield. The next day, the samples were centrifuged at 12000g for 10 minutes at 4°C. The supernatant from each sample was discarded and the RNA pellet was washed with 1mL of 85% ethanol in DEPC treated RNase free water. The samples were centrifuged one final time at 7500g for 5 minutes at 4°C, the supernatant was removed and the RNA pellets were left to air dry. Before the pellets were completely dry, 20µL of DEPC water was added to each

sample and the RNA was dissolved. The quantity of RNA extracted from each sample was determined using a Pharmacia GeneQuant RNA/DNA Calculator. All RNA samples were stored at -80°C.

2.6.2. Reverse Transcription

In order to synthesize cDNA by reverse transcription, total RNA from each sample was used as a template. The reverse transcription reaction, per sample, consisted of 100ng of total RNA, 200u/µl MuLV reverse transcriptase (Applied Biosystems), 25mM $MgCl_2$, 10X PCR buffer, 10mM of dNTPs, 10U/µl RNase Inhibitor and 50µM Random Hexamers, in a final volume of 20 µL. In addition to the reverse transcription reaction for each sample, a control reaction with no MuLV reverse transcriptase was also performed. All the samples, including the control, were processed in a thermocycler (Eppendorf Mastercycler epgradients) at 42°C for one hour then at 95°C for 10 minutes. The cDNA were then stored at -20°C for later use.

2.6.3. Quantitative Polymerase Chain Reaction

Quantitative Polymerase Chain reactions (qPCR) were performed using the cDNA obtained by reverse transcription. For each qPCR reaction 1µL of the cDNA was added to a solution consisting of 1X SYBR Green PCR Master Mix (Qiagen), 0.6µM Forward Primer and 0.6µM Reverse Primer. The samples were processed in a Mx3005 P Real time PCR system (Stratagene). The amplification reactions were done as described: one cycle at 95°C for 15 minutes, followed by forty cycles at 94°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds, ending

with a final cycle at 95°C for one minute, 55°C for 30 seconds and 95°C for 30 seconds. Reading of the sample was done at the end of each of the forty cycles. Forward and reverse primer sets were designed against mouse *cyclophilin b*, *myogenin*, *c-myc*, *p21*, *MyoD, Mef2A, Mef2C* and *p35* (Table 1). Expression levels of *Myogenin, p21*, *Mef2A* and *Mef2C* were normalized to the expression levels of *cyclophilin b* and are represented as percentage for changes over values derived from the pcDNA3 control cells 48 hours after induction of differentiation (48h). Expression levels of *c-myc, MyoD* and *p35* were normalized to the expression levels of *cyclophilin B* and are represented as fold changes over values derived from the pcDNA3 control cells before induction of differentiation (0h).

2.7. Western blotting

2.7.1. Protein extraction

Protein was extracted from the clones #15, #25 and pcDNA3 control, during proliferation and at specific times during differentiation, depending on the experiment. The cells were first rinsed with PBS and then lifted from the dish with a sterile cell scrapers (Corning). The cells suspended in PBS were then transferred to 1.5mL microtubes and centrifuged at 7500 rpm for 5 minutes. The supernatant was removed and the pellet was resuspended in RIPA buffer (25mM Tris-HCl pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS and Complete mini protease inhibitor (Roche)). The samples were then centrifuged at 12000 rpm and the supernatant was transferred to a new 1.5 mL microtube. Cell lysates were stored at -20°C for future western blot analysis. The protein concentrations was determined by BCA assay (Thermo scientific), according the manufacturer specifications.

Name	Primer direction	Primer sequence
cyclophilin B	Forward	5’-GATGGCACAGGAGGAAAGAG-3
	Reverse	5’-AACTTTGCCGAAAACCACAT-3’
myogenin	Forward	5’-CTACAGGCCTTGCTCAGCTC-3’
	Reverse	5’-AGATTGTGGGCGTCGTACG-3’
c-myc	Forward	5’-GCCCAGTGAGGATATCTGGA-3’
	Reverse	5’-ATCGCAGATGAAGCTCTGGT-3’
p21	Forward	5’-CTCTTCTGCTGTGGGTCAGG-3’
	Reverse	5’-AAGTTTGGAGACTGGGAGAG-3’
MyoD	Forward	5’-ACTTTCTGGAGCCCTCCTGGC-3’
	Reverse	5’-TTTGTTGCACTACACAGCATG-3’
Mef2A	Forward	5’-GAATGCCCAAAGGATAAGCA-3’
	Reverse	5’-CAGCATTCCAGGGGAAGTAA-3’
Mef2C	Forward	5’-ATCTGCCCTCAGTCAGTTGG-3’
	Reverse	5’-CAGCTGCTCAAGCTGTCAAC-3’
p35	Forward	5’-AGCCCTTCCTGGTAGAGAGC-3’
	Reverse	5’-CCTGACCGCTCTCATTCTTC-3’

Table 1: List of primers used for qRT-PCRs.

In order to detect mouse *cyclophilin b*, *myogenin*, *c-myc*, *p21*, *MyoD, Mef2A, Mef2C* and *p35* we generated primers using the sequences found in the National Center for Biotechnology Information database and a primer design software, Primer3.

2.7.2. Western blot

Depending on the experiment, 15 to 30 µg of protein from each sample was transferred to new microtubes to which 1X protein loading buffer (62.5mM Tris-HCl pH 6.8, 2% SDS, 20% glycerol, 5% β-mercaptoethanol and 0.02% bromophenol) was added. Samples were then boiled for 5 minutes at 100°C and left to cool. Proteins were subsequently resolved, at 100V, in either 8% or 6% SDS-polyacrylamide minigels (SDS-PAGE) depending on the size of the protein of interest. The proteins were transferred overnight at 30V or for one hour at 100V on polyvinylidene fluoride (PVDF) or Nitrocellulose membranes (Millipore), depending on the experiment. Following transfer, Membranes were blocked with 5% non-fat skim milk in PBS with 0.05% tween-20 (PBST) for 30 minutes and then probed overnight at 4°C or for one hour at room temperature with a specific primary antibody diluted in 1% skim milk PBST. The primary antibodies used were: a 1:1 dilution of F5D hybridoma supernatant (myogenin), a 1:1 dilution of MF20, a 1:2500 dilution of mouse anti-HA.11 (Covance, 16B12), a 1:1000 dilution of mouse anti-c-myc (ab11917, Abcam) and a 1:2000 mouse anti-MyoD (MoAb 5.8A, BD Biosciences). The blots were then washed three times for 10 minutes with PBST and incubated at room temperature with either a 1:10000 dilution of Goat anti-mouse, Immunoglobulin G (IgG) , horseradish peroxidase (HRP) conjugated secondary antibody in PBST-1% skim milk or a 1:10000 mouse anti-β-actin IgG HRP (sc-47778, Santa Cruz). The membranes were washed three more times with PBST and incubated with Western lightning ECL (Perkinelmer) as specified by the manufacturer. The proteins were detected by chemiluminescence using CL-XPosure film (Thermo scientific), which was then developed using a Kodak X-OMAT 200A processor. Protein levels were determined using the ImageJ software and were normalized with the housekeeping gene, β-actin. They were represented as percentage fold changes over values

derived from the pcDNA3 control cells 48 hours after induction of differentiation (48h) for myogenin and MyHC or were represented as fold changes over values derived from the pcDNA3 control cells before induction of differentiation (0h) for Staufen1-HA, c-myc and MyoD.

2.8. Wound healing assay

Stable clones and a pcDNA3 control cells were seeded at the same density in six well plates. Once the cells have reached confluency, the monolayer was scratched using the tip of a 200µL pipette tip to form a cross shaped wound. The plates were then washed with sterile PBS to remove cellular debris, fresh GM was added and the cells were incubated at 37°C in 5% CO_2. Pictures were taken at 10X magnification with a Zeiss Axiovert S100 2TV inverted microscope, at the time of the creation of the wound and every 2 hours for 8 hours, at the same location for each wound. Software specially designed to analyze Wound Healing assays, called tscratch, was used to analyze the pictures and determine the percentage of wound area at each of the time points. The ability of cell to migrate into the wound area was assessed by comparing pictures taken at 2 hour intervals of the initial injury (0h), for 8 hours, and comparing them with the first pictures taken. The percentage of non-recovered wound area was calculated by dividing the wound area at each of the time points after injury, by the initial wound area. The rate of migration was calculated by subtracting the percentage distance between the edges of the wound area, right after creation of the wound (100%), by the percentage after 8 hours and dividing it by the number of hours that had passed (8h).

2.9. BrdU myoblast proliferation assay

Stable clones #15, #25 and a pcDNA3 control were seeded on glass coverslips at low density and were left to proliferate overnight in GM. The next morning proliferation capacity of each clone was assessed using the 5-Bromo-2'-deoxy-uridine Labeling and Detection Kit I (Roche). The GM was removed and the cells were incubated for 2 hours with 5-Bromo- 2'-deoxy-uridine (Roche), known as BrdU, at a final concentration of 10µM BrdU/mL of fresh GM. The media was then aspirated, the cells were rinsed with 1X Washing Buffer (Roche), fixed with Ethanol Fixative (0.563g glycine, 150mL ddH2O, 350 mL, pH 2.0) for 20 minutes at -20°C and washed three additional times with 1X Washing Buffer. Afterwards, the cells were incubated for 30 minutes at 37°C with Anti-BrdU working solution (Roche) and then washed three more times with 1X Washing Buffer. The cells were subsequently incubated with Anti-mouse-Ig-fluorescein working solution (Roche) for 30 minutes at 37°C and washed three final times with 1X Washing Buffer. The cover slips were then mounted onto glass slides with Vectashield mounting medium containing DAPI (Vector laboratories Inc.). The BrdU labeled myoblasts, were observed through a Zeiss 2.5X objective with a Zeiss Axioskop2 mot plus instrument. Images were captured through different fluorescent filters using a Qimaging Micropublisher digital imaging system and merged with the Northern Eclipse software.

2.10. MyoD add-back experiment

Clones #15, #25 and pcDNA control cells were seeded at the same density on Matrigel coated 35 mm plates and left to proliferate in GM overnight. The next morning the cells were transiently transfected with a MyoD containing plasmid or an empty vector (CTL) as negative

control, following the same protocol as described previously. The cells were left to proliferate once more overnight. The next day, the cells had reached 90% confluency and were induced to differentiate for 48 hours. Total protein was extracted from the cells at 90% confluency (0h), 24h and 48h after induction of differentiation and western blots to detect MyoD, myogenin and β-actin were performed as described previously. The myotubes were visualized by immunohistochemistry, 72 hours after induction of differentiation, using a MF20 primary antibody to detect MyHC, as described previously.

2.11. c-myc long 5'UTR-CAT plasmid construction

2.11.1. Design of the c-myc long 5'UTR primers

The sequence of the mouse c-myc long 5'UTR (NC_000081.5) was obtained through the National Center for Biotechnology Information (NCBI) database. Using the primer design software Primer3 (v. 0.4.0), forward and reverse primers were designed, in order to obtain as much of the sequence of the c-myc long 5'UTR as possible: F=5'-CCCACCCGCCCTTTATATT-3' and R=5'-CGTCGTGGCTGTCTGCGG-3'.To ensure that the sequence would be inserted into the plasmid in the right orientation, two different restriction sites, KpnI and SalI, were added to the 5'end of the forward and reverse primer: F=5'-GGTACCCCCACCCGCCCTTTATATT-3' and R=5'-GTCGACCGTCGTGGCTGTCTGCGG-3'. In order for the restriction reaction to occur properly, a random sequence of four nucleotides was added to the 5'end of each primer: F=5'-*ATAT*GGTACCCCCACCCGCCCTTTATATT-3' and R=5'-*ATAT*GTCGACCGTCGTGGCTGTCTGCGG-3'. Once the design process was completed, the primers were ordered form Sigma Aldrich.

2.11.2. c-myc 5'UTR PCR amplification

To obtain the full sequence of the c-myc 5'UTR, it was necessary to perform a PCR reaction, using the primers designed previously and cDNA that contained c-myc mRNA. The PCR was prepared by adding 1μL of the random cDNA samples, obtained from previous experiments, to a PCR master mix (25mM $MgCl_2$, 10X PCR buffer, 2mM dNTPs, 10μM Forward primer, 10μM Reverse primer and 5U/μL Taq DNA Polymerase (Fermentas)). The samples were then processed in a thermocycler (Eppendorf Mastercycler epgradients) where a standard PCR reaction was performed: The Taq DNA Polymerase was first activated 95°C for 2 minutes, this was followed by 38 amplification cycles at 95°C for 30 seconds, at 60°C for 30 seconds and at 72°C for 30 seconds and a final cycle at 72°C for 10 minutes at the end of the 38 cycles. The amplified products were purified by electrophoresis in a 1% agarose gel. Three bands, corresponding to three products of different size, were obtained. Each DNA products was extracted from the gel using a QIAquick Gel extraction kit (Qiagen), according to the manufacturers' specifications. They were then sent to the Ontario Genomic Innovation Center (Ottawa Hospital Research Institute, StemCore Laboratories, Ottawa, On, Canada) for sequencing, where it was determined that the largest of the three products was the c-myc long 5'UTR.

2.11.3. Digestion and ligation

To obtain a compatible fragment that could be inserted into the KpnI and XhoI restriction sites of the CAT reporter plasmid, 1μg of the c-myc long 5'UTR was first incubated for one hour

at 37°C with 0.5µL of KpnI restriction enzyme (Fermentas) in a solution containing 2µL of KpnI buffer (Fermentas) and 16.5µL of DEPC water. After that, 1µL SalI restriction enzyme (Fermentas), 4 µL of Orange buffer and 15µL of DEPC water was added to the reaction mix and incubated for another hour at 37°C. At the same time, the empty CAT reporter plasmid was double digested with 0.5µL of the restriction enzymes KpnI and XhoI (Fermentas) in a solution containing 2µL of Tango buffer (Fermentas) and 16.5µL of DEPC water. Once the digestions were complete the plasmid was dephosphorylated and both the plasmid and insert were purified by phenol/chloroform purification. Subsequently, a ligation was performed by adding 1µL of purified plasmid and 7µL of the c-myc long 5'UTR to 1µL of 10X ligase buffer (Fermentas) and 1µL of T4 DNA ligase (Fermentas) and incubating the mix for one hour at room temperature.

2.11.4. Transformation

Once the ligation was complete, the entire 10µL ligation mix was added to 200µL of competent DH5β E.coli and left on ice (4°C) for 30 minutes. Next, they were heat shocked for 45 seconds at 42°C and then left on ice (4°C) for 5 minutes. In order to stimulate Ampicillin resistance gene expression, 1mL of SOC media (2% Tryptone, 0.59% Yeast Extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ and 20mM glucose) was added to the 1.5 mL microtubes containing the bacteria. The cells were then incubated on a shaker for one hour at 37°C. The transformed bacteria were then spread onto a sterile ampicillin containing agar plate (20g/L premix LB agar (Wisent) and 100µL/mL ampicillin) and incubated overnight at 37°C. The next morning, six individual colonies were isolated and were cultured overnight in 5mL of sterile LB media (1% Tryptone, 0.5% Yeast Extract, 0.5% NaCl). The plasmid DNA was

extracted from each colony using a QIAPrep Spin Minipre Kit (Qiagen), following the manufacturers specifications. To determine if the clones isolated possessed the insert a double digestion was performed: 1µg of plasmidic DNA obtained from each clone was incubated for one hour at 37°C with 0.5µL of the restriction enzymes, XhoI and PstI (Fermentas), in a solution containing 2µL of Red buffer (Fermentas) and 15.5µL of DEPC water. After digestion, the DNA was resolved by electrophoresis in a 1% agarose gel and, as predicted, two fragments were obtained for all the clones: meaning that the ligation was successful. To confirm this, the plasmidic DNA from one of the clones was sent to the Ontario Genomic Innovation Center (Ottawa Hospital Research Institute, StemCore Laboratories, Ottawa, On, Canada) for sequencing. It was determined that the ligation was successful and that the clone tested possessed the c-myc long 5'UTR-CAT expressing construct. This clone was amplified overnight in 250mL of sterile LB. The next day the plasmidic DNA was extracted using a Highspeed Plasmid Maxi Kit (Qiagen), following the protocol provided by the manufacturer. The construct was then stored at -20°C.

2.12. CAT assay

Clones #15, #25 and a pcDNA3 control were seeded at low density (<30%) on 35mm plates and left to proliferate overnight in GM. The next morning the cells were transiently transfected, as described previously, with either an empty CAT reporter plasmid or the c-myc long 5'UTR-CAT reporter construct. One day after transfection, the GM was aspirated and the cells were washed twice with PBS. Afterwards, 200µL of 1X Passive lysis buffer (Promega) was added to each well and then the cells were incubated for 10 minutes at 37°C. The cells were then scraped

with a cell scraper and the content of each well was put into individual 1.5 mL microtubes. The sample were frozen and thawed repeatedly to further lyse the cells. After which, the microtubes were centrifuged at 12000rpm for 10 minutes, the supernatant was transferred to a new microtube. At this point, 50µL of the lysate was heated at 60°C for 10 minutes. Negative and positive controls were also heated: the first contained only 1X Passive lysis buffer while the second was made up of 0.25µL of Chloramphenicol Transferase in 50µL of 1X Passive lysis buffer. A master mix was then prepared containing 3µL of ^{14}C Chloramphenicol (Perkin Elmer), 5µL of Butyryl CoA (Sigma Aldrich) and 67µL of DEPC, per sample to be tested. After the 10 minutes of incubation, 75µL of Master Mix was added to each lysate, the positive and the negative control. They were then incubated for 2 hours at 37°C, after which 300µL of xylene was added to each sample to stop the reaction. The lysates were then vortexed for 30 seconds and centrifuged at 15000rpm for 3 minutes at room temperature. 190µL of supernatant was transferred to a new microtube tube and one volume of 0.25M Tris-HCl pH8 was added. The samples were once again vortexed for 30 seconds and centrifuged at 15000rpm for 3 minutes at room temperature. This time, 160µL of supernatant was transferred to a new centrifuge tube and one volume of 0.25M Tris-HCl pH8 was added. This sequence of steps was repeated one last time and then 120µL of the supernatant from each sample was added to 2mL of scintillation fluid, in a scintillation vial. The vials were placed in a Wallac WinSpectral 1414 Liquid Scintillation counter and ^{14}C activity was detected.

3. Results

3.1. Generation of a stable C_2C_{12} cell lines over-expressing mouse Staufen1-HA

To explore the functional role of Staufen1 in C_2C_{12} differentiation, we established C_2C_{12} myoblasts that stably over-expressed Staufen1. Thirty-six clones of G418 resistant myoblasts were initially isolated after having undergone two or three weeks of drug selection. We determined, by western blotting, that twelve of these appeared to stably over-express the Staufen1-HA construct: clones #3, #5, #11, #12, #14, #15, #18, #19, # 22, #25, #31, and #32 (Figure 5A). However, after a few more weeks of selection we discovered, by fluorescence microscopy, that we had lost the expression of our Staufen 1-HA in a number of these clones and only five of those twelve clones still expressed our construct: clones #3, #11, #14, #15 and #25 (Figure 5B). Two independent clones over-expressing Staufen1, clones #15 and #25, were analyzed, though all the other Staufen1 over-expressing clones have been tested and identical results were found. Cells expressing empty vector, selected in the same manner as the Staufen1 over-expressing stable cell lines, were used as control and named pcDNA3. As shown in Fig.5C, during proliferation, clones #15 and #25 express the Staufen1-HA construct while the pcDNA3 control does not.

3.2. Mouse Staufen1 over-expression negatively effects C_2C_{12} differentiation

Clones #15 and #25 were seeded at the same density as the pcDNA3 control and once they reached 90% confluency were then allowed to differentiate. At different time points during differentiation, pictures were taken and analyzed to determine the ability of these cells to

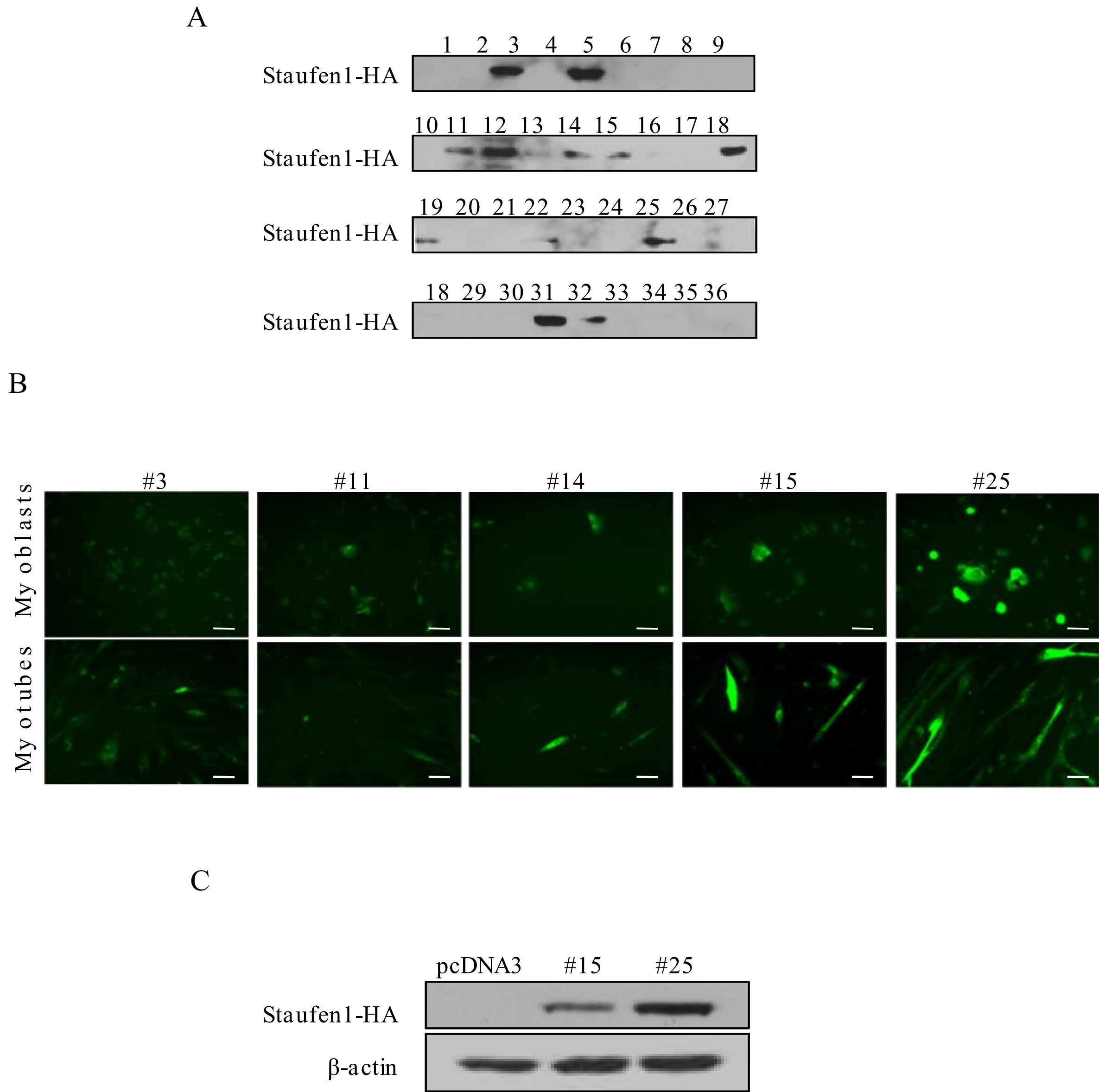


Figure 5: **Generation of the Staufen1-HA over-expressing stable C_2C_{12} cell lines.**

C_2C_{12} myoblast were transfected with a pcDNA3-Staufen1-HA construct, selected for several weeks, after which isolated clones were harvested. (A) Total protein was extracted from thirty-six clones isolated and myogenin was detected by western blotting. (B) Staufen1-HA was detected by immunohistochemistry, in clones #3, #11, #14, #15 and #25, during proliferation and after four days of differentiation. Pictures were taken at 10x and 2.5x magnification for Myoblasts and Myotubes, respectively. Bar, 20µm. (C) Total protein was extracted during proliferation from clones #15, #25 and the pcDNA3 empty vector control. Western blots were performed to detect Staufen1-HA and β-actin, which was used as loading control.

undergo myogenic differentiation. As shown in Fig.6, after 24 hours of differentiation, small differences can be seen between the pcDNA3 cells and the two Staufen1 over-expressing stable clones: small mononucleated myotubes can be seen in pictures from the pcDNA3 control but not in those of clones #15 and #25. Once the cells had been differentiating for 72 hours, we noticed that the myotubes formed by the clones #15 and #25 were smaller than those of the pcDNA3 control. From these initial observations, we concluded that Staufen1 over-expression in C_2C_{12} cells did not completely inhibit differentiation but rather delayed the formation of myotubes.

One possible explanation for this is that expression levels of our Staufen1-HA construct somehow decreased during differentiation. To find out if the expression levels of the Staufen1-HA construct changes during differentiation of clones #15 and #25, we performed western blots using a mouse anti-HA primary antibody. We observed that Staufen1-HA expression, in our stable clones, decreases during differentiation (Figure 7A and B). One possible explanation for this is that promoter driving Staufen1-HA expression is an RSV promoter which is sensitive to serum levels. When high serum GM is changed for low serum DM, RSV promoter activity is reduced leading to an overall decrease of the protein whose expression is driven by this promoter.

3.3. Staufen1 over-expression affects myoblast fusion and differentiation

To quantify the differences observed in myotube size, the clones #15, #25 and pcDNA3 were differentiated for 96 hours. Cells were then fixed and immunostained for MyHC as described in

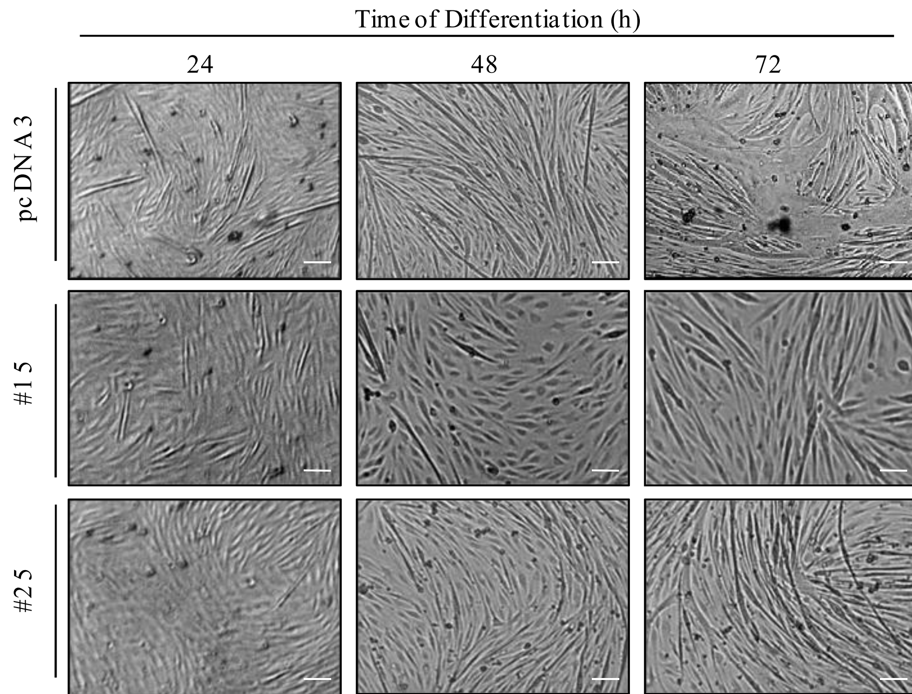


Figure 6: **Staufen1 over-expression delays differentiation.**

Clones #15 and #25 and the pcDNA3 control cells were left to proliferate in growth media (GM) until they reached 90% confluency, at which time differentiation was induced by serum deprivation. 24 hours after induction of differentiation phase contrast pictures of a single representative field of view are shown. 48h and 72h after induction of differentiation 2.5x magnification phase contrast pictures are shown. Bar, 75µm.

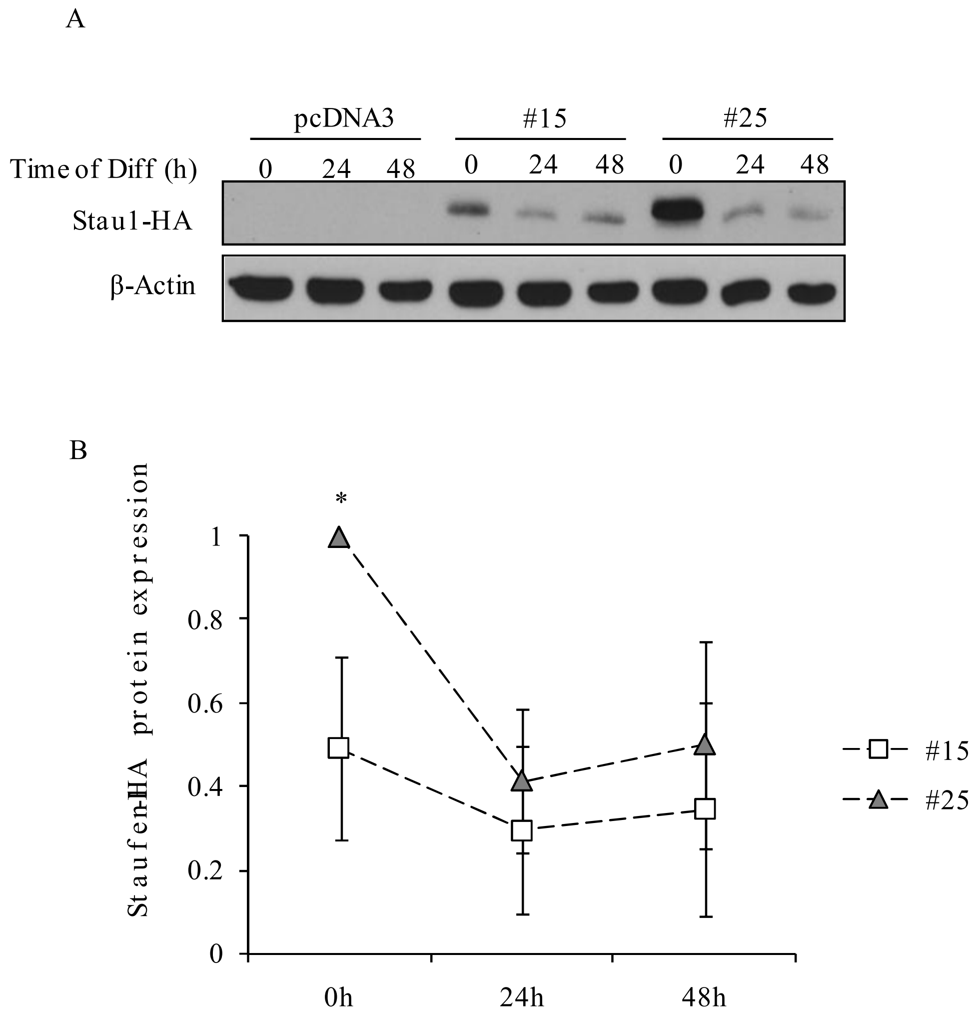


Figure 7: **Staufen1-HA expression decreases during differentiation**.

Staufen1 over-expressing stable clones #15 and #25 and the pcDNA3 empty vector control, were seeded at the same density on matrigel coated 35mm plates. Once they had reached 90% confluency, differentiation was induced. Total protein was extracted upon induction (0h), 24 hours and 48 hours after induction and used for Western blotting. (A) PVDF membranes were probed for Staufen1-HA and β-actin. Staufen1-HA protein levels were normalized with β-actin and (B) the amount of Staufen1-HA at each time point was represented relative to clone #25 before induction of differentiation (0h). Results are mean ± SE (n=3); *p<0.05.

materials and methods (Figure 8A). From the pictures that were taken, we calculated the differentiation index (DI) (percentage of nuclei in MyHC positive cells), the fusion index (FI) (percentage of nuclei in MyHC positive cells with three of more nuclei) and the average number of nuclei per myotube and found that in all three cases there was a significant difference between the pcDNA3 cells and the clones #15 and #25 ($p<0.001$), see Fig.8 A to C.

Even though the decrease of the DI and FI was shown to be statistically significant, it did not completely reflect what we could observed from the pictures. This was due to the fact that calculation of the fusion and differentiation indices takes into account the total number of nuclei and that there are fewer nuclei in the pictures taken of the clones #15 and #25 compared to the ones taken from the pcDNA3 control (Figure 8D). Whereas the pcDNA3 control harbored on average 1265 nuclei ± 30 per picture, the Staufen1 over-expressing clones #15 and #25 displayed only 780 ±39 and 648 ±38 nuclei per picture respectively. This difference in the total number of nuclei could be caused by a decrease in proliferation or an increase in apoptosis or necrosis during differentiation when Staufen1 is over-expressed. To circumvent this problem, the average number of nuclei per myotube was calculated for each clone. As shown in Fig.8E, there was a greater difference between the pcDNA3 control and the clones #15 and #25, which was more representative of what we could observe in the pictures. With the pcDNA3 cells having an average of 139 nuclei per myotube ±13 nuclei compared to the clones #15 and #25 which only have 40 ±4 and 9 ±1 nuclei per myotube. Thus, our results confirm that the fusion and differentiation processes are severely affected in the C_2C_{12} cells stably over-expressing Staufen1.

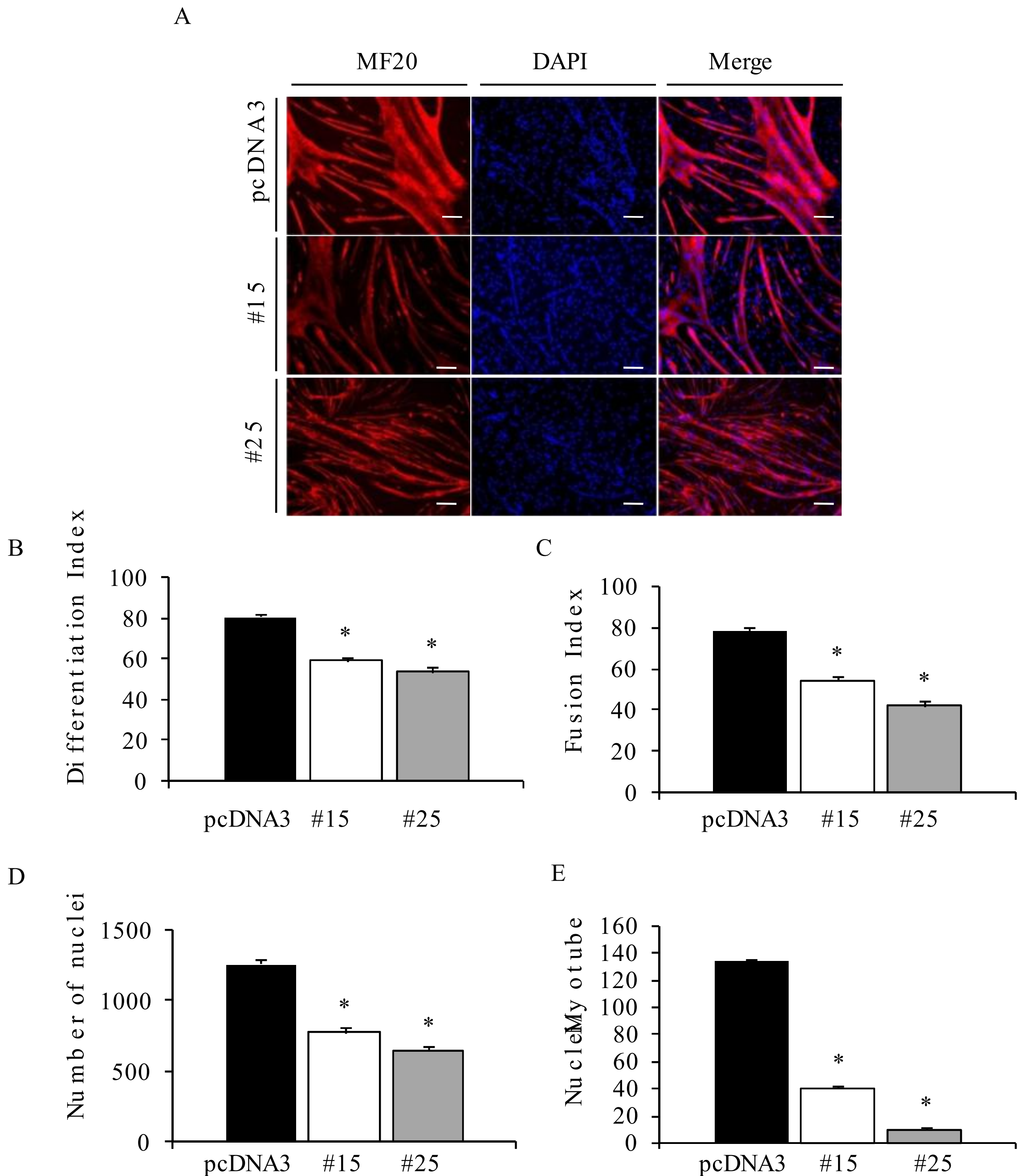


Figure 8: **Staufen1 over-expression affects proper myotube formation.**

Quantitative analysis of the fusion and differentiation potential of the Staufen1-HA over-expressing clones, #15 and #25, and the pcDNA3 control cells. (A) Pictures of MF20 (MyHC) and DAPI double labeled cells were taken four days after induction of differentiation, at 2.5x magnification. Bar, 75µm. From these, (B) the differentiation index (% of nuclei in MyHC positive cells), (C) the fusion index (% of nuclei in MyHC positive cells with three or more nuclei), (D) the total number of nuclei and (E) the average number of nuclei per myotube were calculated. Results are mean ± SE (n=4); *$p<0.001$

Myoblast migration plays an import role in C_2C_{12} differentiation and more specifically in the fusion process, since it enables myoblasts to align and subsequently fuse to form multinucleated myotube. To examine the effects of Staufen1 over-expression on C_2C_{12} cell migration, we performed wound-healing assays using the clones #15, #25 and a pcDNA3 (Figure 9A). The difference between the percentages of the distance of cell migration relative to the initial distance between the wound edges directly after scratching the monolayer was more significant after 8 hours (Figure 9B). At this time point, we observed that the pcDNA3 cells have a significantly higher percentage of recovered wound area compared to the clones #15 and #25 ($p<0.05$), whereas the clones #15 and #25 were not significantly different from one and other. From these values we were able to determine the average rate of cell migration (% of recovered wound area per hour), for each clone, and found that it was significantly decreased in the Staufen1 over-expressing C_2C_{12} cells compared to the pcDNA3 control (Figure 9C). All these observation indicate that Staufen1 over-expression appears to slow C_2C_{12} cell migration *in vitro* which could possibly lead to a decrease in myoblast fusion.

3.4. Staufen1 over-expression affects the expression of the myogenic markers: myogenin and MyHC.

Since the DI, FI and the average number of nuclei per myotube, were significantly lower in the Staufen1 over-expressing cell lines compared to our control, we wondered whether the expression of muscle specific genes was affected. We therefore looked at the expression of an early and a late marker of differentiation: myogenin, a bHLH MRF that is up-regulated directly

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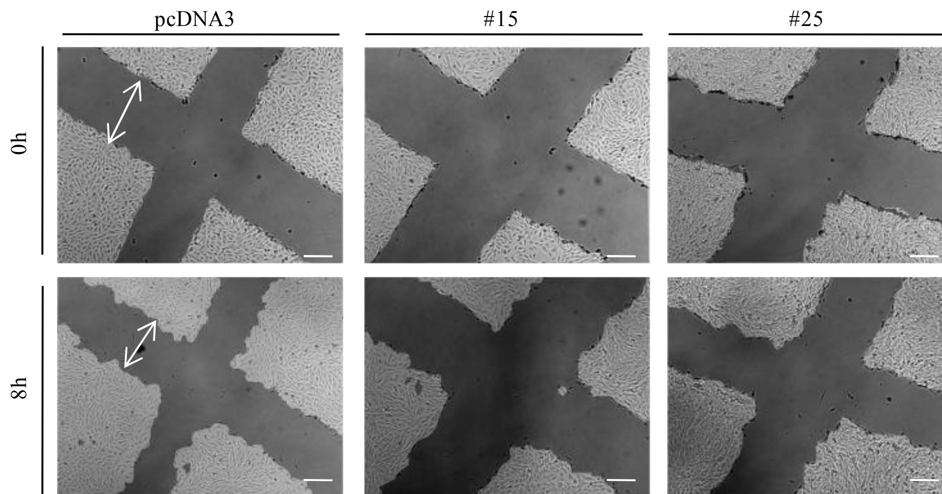

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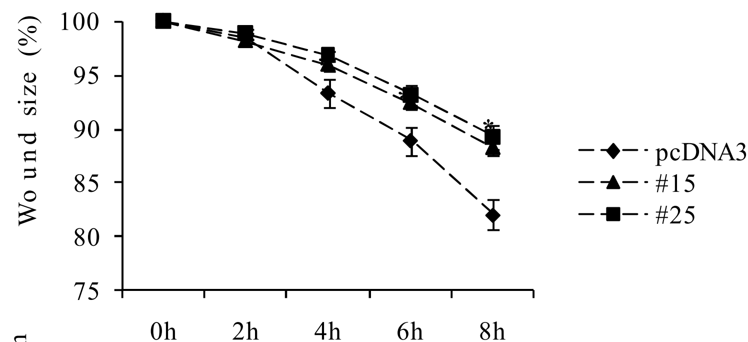

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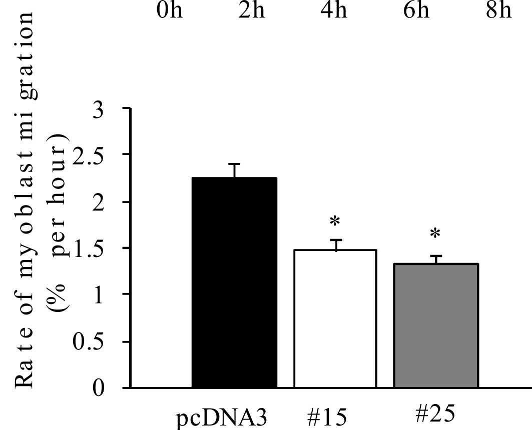

Figure 9: **Staufen1 over-expression decreases myoblast motility**.

The Staufen1 over-expressing stable clones #15 and #25, and pcDNA3 empty vector control cells were seeded at the same density in six well plates. Once they reached confluency, Wound Healing assays were performed over an 8h period. (A) Pictures of the cross shaped wounds were taken immediately after injury (0h), 2h, 4h, 6h and 8 hours after injury, at 10x magnification. Bar, 75µm. (B) The percentage of non recovered wound area was calculated, for all of these time-points, using the pictures and the tscratch software. (C) The rate of myoblast migration into the wound (percentage of recovered wound area per hour) was determined, for each clone. Results are mean ± SE (n=3); *p<0.05.

after induction of differentiation and MyHC which is up-regulated after 24 hours of differentiation.

As predicted, we found that the pcDNA3 control cells displayed the classical increase in myogenin and MyHC protein at 90% confluency (0h) and after 48 hours of differentiation (Figure 10A). On the other hand, we found that the appearance of both myogenin and MyHC was delayed in the clone #15 and #25 compared to the pcDNA3 control cell line (Figure 10A).

We quantified, using the ImageJ software, the intensity of the bands at each time point from multiple western blots and observed that there was a statistically significant decrease, in clones #15 and #25, of the protein expression of both these markers at 90% confluency (0h), 24h and 48h after induction of differentiation compared to the pcDNA3 cells ($p<0.05$) (Figure 10B). Once the cells had reach 90% confluency we can already observe the expression of myogenin protein in pcDNA3 cell, whereas, at this point clone #25 did not express any myogenin protein, and clone #15 expressed levels that were 96% lower than the pcDNA3 control. We then found that myogenin protein levels were 84% and 89% lower 24 hours after induction, in the clones #15 and #25 respectively, compared to control levels. Finally, by 48 hours myogenin protein levels were 56% and 60% lower in clones #15 and #25 compared to control. As for MyHC, we observed that it was expressed at low levels in the pcDNA3 control, 24 hours after induction of differentiation, but was not expressed at all in the clones #15 and #25 until 48 hours after induction. We therefore quantified the difference between these clones and the pcDNA3 control cell at this time point. We found that the protein expression was 97% and 93% lower in the clones #15 and #25 compared to that of control ($p<0.001$) (Figure 10C). The delay and decrease protein expression of both these markers suggest that Staufen1 affects one of more protein necessary for the activation of the myogenic program.

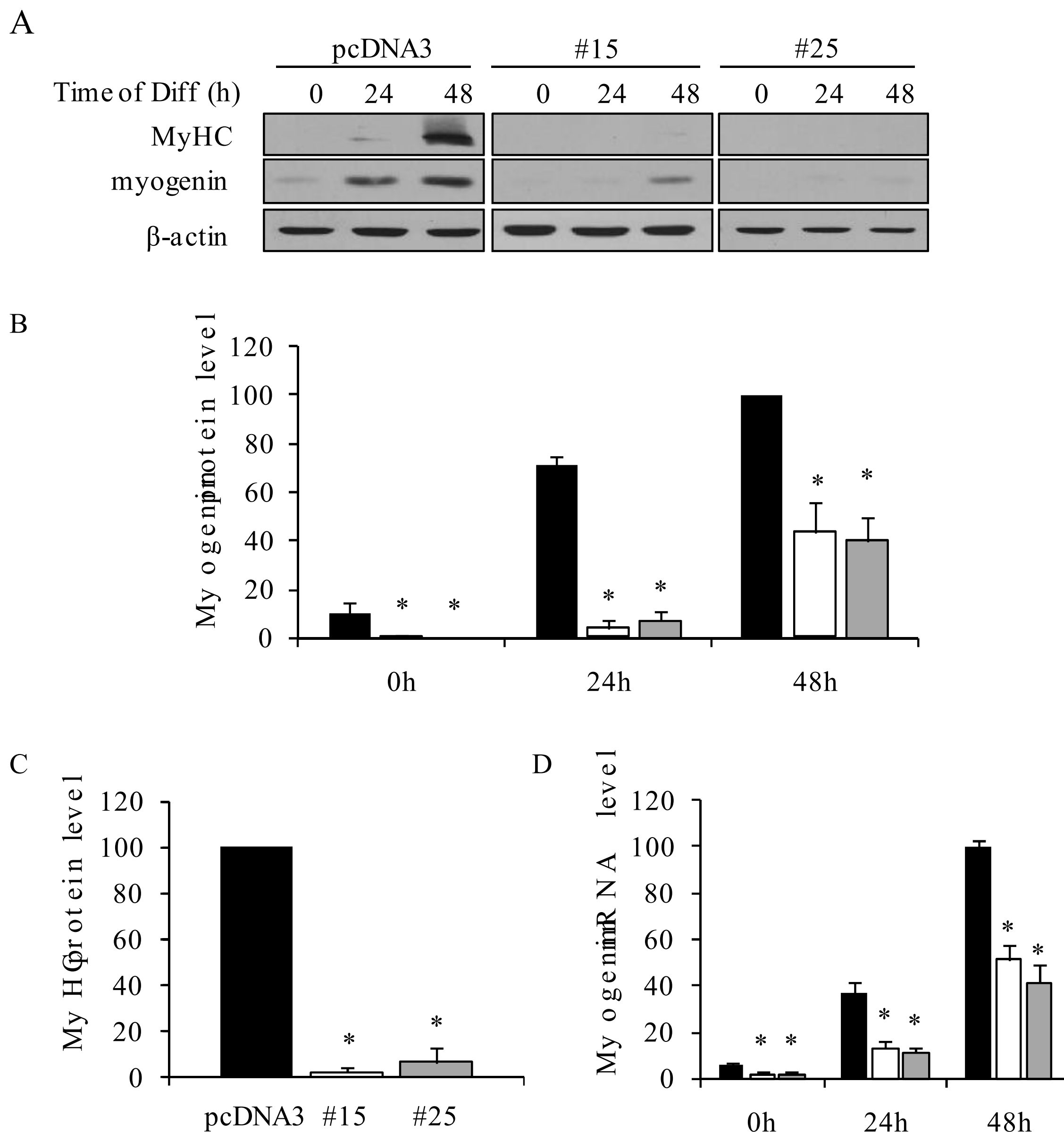

Figure 10: **Staufen1 over-expression delays the expression of myogenic markers myogenin and MyHC during differentiation.**

Clones #15 and #25 and the pcDNA3 control cells were left to proliferate in growth media (GM) until they reached 90% confluency, at which time differentiation was induced by serum deprivation. Total protein and RNA was extracted upon induction (0h), 24h and 48h after induction of differentiation. (A) MyHC, myogenin and β-actin proteins were detected by Western blotting. Protein levels were normalized with β-actin. (B) MyHC levels were represented at the 48h time point. Results are mean ± SE (n=3); *$p<0.001$. (C) Myogenin protein levels were represented at 0h, 24h and 48h after induction of differentiation. Results are mean ± SE (n=5); *$p<0.001$. (D) *Myogenin* mRNA levels were quantified by qRT-PCR relative to *cyclophilin B* mRNA levels. Results are mean ± SE (n=5, duplicates); *$p<0.001$. All the values are represented as percentage fold changes over values derived from the pcDNA3 control, 48h after induction of differentiation.

As it was mentioned previously, the increase in myogenin protein expression during differentiation is caused, for the most part, by an increase in myogenin transcription. We therefore wanted to confirm that the delay observed in myogenin protein expression was due to a decrease in transcription. Similarly to what was observed at the protein level, we found a significant difference in myogenin mRNA expression in clones #15 and #25 compared to the pcDNA3 control throughout differentiation ($p<0.05$). As shown in Fig10D, myogenin mRNA expression in clones #15 and #25, at 90% confluency, were 37% and 39% lower than those of pcDNA3 cells. Once the cells were differentiated for 24 hours, the myogenin mRNA levels of clones #15 and #25 were 36% and 30% lower than those of control cells and finally, 48 hours after induction of differentiation the levels were 51% and 41% lower in the clones #15 and #25 than in the pcDNA3 control. This decrease in myogenin mRNA transcription would indicate that the Staufen1 target or targets are upstream of the activation of myogenin transcription during skeletal muscle differentiation.

It has been established that passaging C_2C_{12} cells can, if not done properly, result in the selection of a subpopulation of myoblasts that differentiate poorly. This can occur when C_2C_{12} cells are left to proliferate until they become confluent and start to differentiate. If the cells are passaged at this point, only the ones that have not started differentiating will survive and continue to proliferate, thus selecting myoblasts that differentiate poorly. It is therefore possible that the results that we have obtained are the result of negative selection. It is also possible that what we have observed is due to a clonal effect, meaning that in our selected clones, our Staufen1-HA construct could have inserted itself in a gene that is necessary for differentiation, leading to the delay observed. In order to rule out these two possibilities, we transiently

transfected C_2C_{12} cells with the same two plasmids that were used to generate the stable cell lines, as described in materials and methods. Once the transfection was complete and the cells had sufficient time to express the construct, differentiation was induced. Protein and mRNA was extracted before induction (0h), 24h and 48h after induction of differentiation. Western blots and quantitative RT-PCRs were performed on these samples to look at myogenin protein and mRNA expression. In both cases we obtained similar results to the ones obtained with the Staufen1 over-expressing clones shown in Fig.11. We observed that the myogenin mRNA levels at 90% confluency and 24 hours after induction of differentiation, were significantly decreased in the Staufen1-HA transfected cells compared to empty vector control ($p<0.05$) Fig.11A. As for myogenin protein levels, they were delayed and significantly decreased 24 hours and 48 hours after induction of differentiation, in the C_2C_{12} cells transfected with the Staufen1-HA construct compared to those transiently transfected with the empty vector ($p<0.5$) Fig.11B. These results confirmed that our previous results were not due to negative selection or clonal effects and that they were truly a consequence of Staufen1 over-expression.

3.5. Staufen1 over-expression affects the expression of the myogenic regulatory factor and enhancers: MyoD, Mef2A and Mef2C.

We have shown that myogenin mRNA expression is decreased early in the differentiation process leading to a decrease in myogenin protein levels (Figure 10B and D). According to the literature, this decrease in myogenin alone could explain the delay observed in MyHC protein

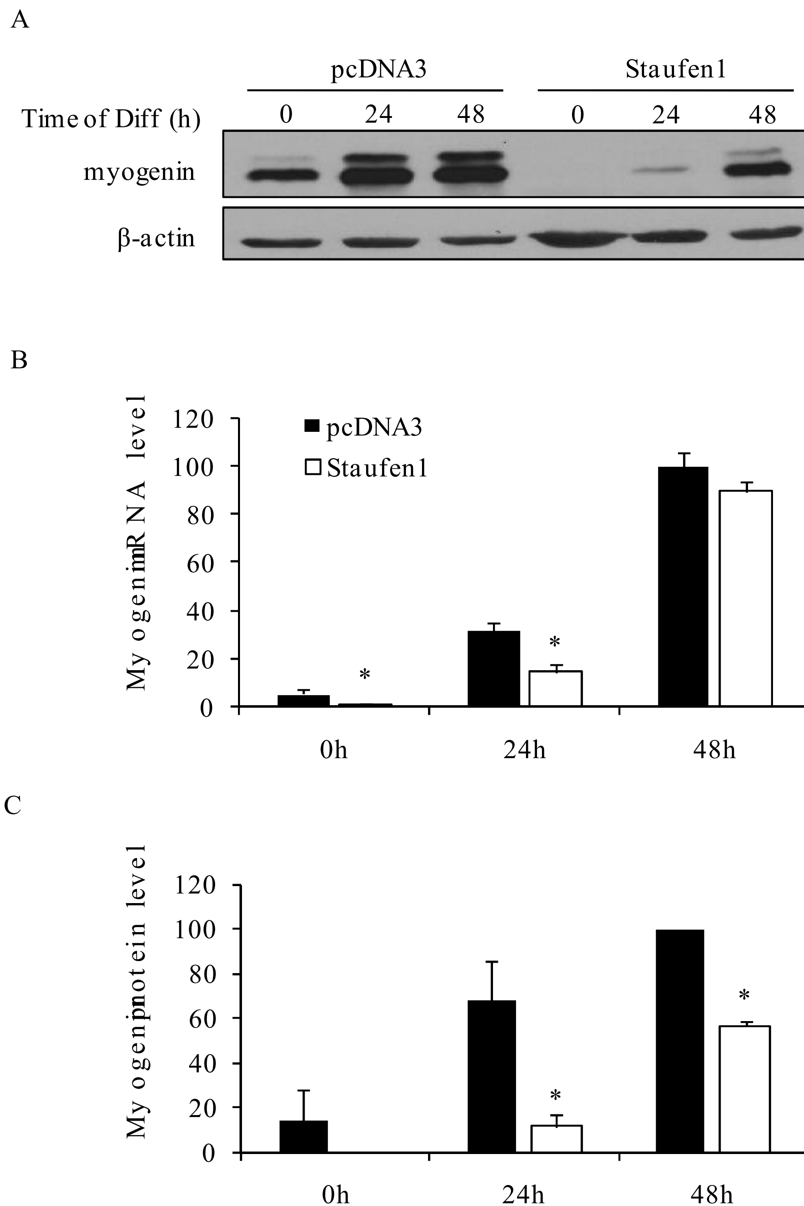

Figure 11: **Transient transfection of Staufen1-HA induces a delay in myogenin expression.**

C_2C_{12} cells were transiently transfected with a pcDNA3-Staufen1-HA construct or an empty pDNA3 vector and were left to proliferate in growth media. Once they reached 90% confluency, differentiation was induced. Total protein and RNA was extracted at the moment of induction (0h), 24h and 48h after induction. (A) Myogenin and β-actin proteins were detected by Western blotting. (B) *Myogenin* mRNA levels were quantified by qRT-PCR relative to *cyclophilin B* mRNA levels. Results are mean ± SE (n=3, duplicates); *p<0.05. (C) Myogenin protein levels were quantified relative to β-actin. All the values are represented as percentage fold changes over values derived from the pcDNA3 control, 48h after induction of differentiation. Results are mean ± SE (n=3); *p<0.05.

expression. In order to narrow down the Staufen1 target or targets that are responsible for the delays observed, we looked at the expression of one of the main proteins responsible for increasing myogenin transcription during differentiation: MyoD. As shown in Fig.12B and Fig.12C, we found that during proliferation (0h) and differentiation, the expression of both MyoD protein and mRNA was, for the most part, significantly reduced in the Staufen1 over-expressing clones #15 and #25 compared to the pcDNA3 control ($p<0.05$). From this, we can conclude that the hallmark increase in MyoD protein, that drives the differentiation process once the growth factors are removed from the media, does not occur as it should in our Staufen1 over-expressing clones, which would explain the delay and decrease in the myogenic markers myogenin and MyHC.

It is important to note that MyoD expression alone cannot drive the differentiation cascade but requires the presence and upregulation of factors such as the Mef2 family of MADS box transcription factors which is made up of four members Mef2A, Mef2B, Mef2C and Mef2D (Molkentin et al., 1995). Furic et al. (2008) found that Mef2A mRNA was present in Staufen1^{55}-HA containing mRNPs, which could indicate that Staufen1 binds Mef2A. This lead us to hypothesize that Mef2A could be a possible SMD target that would be responsible for the delay observed in differentiation upon Staufen1 over-expression. We looked at the Mef2A mRNA levels in the Staufen1 over-expressing clones #15 and #25 and compared them to those in the pcDNA3 control cells. We found that at 90% confluency the Mef2A mRNA levels, in clones #15 and #25, were significantly lower than those of the pcDNA3 control ($p<0.05$) (Figure 13A). This significant decrease was also observed 24h and 48h after induction of differentiation ($p<0.05$).

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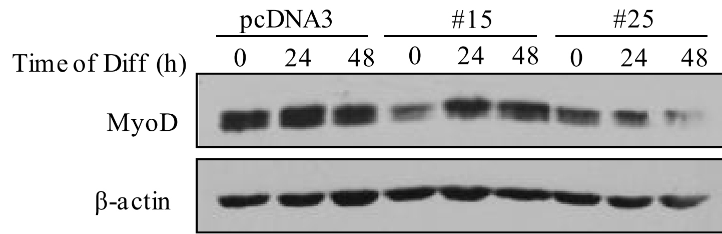

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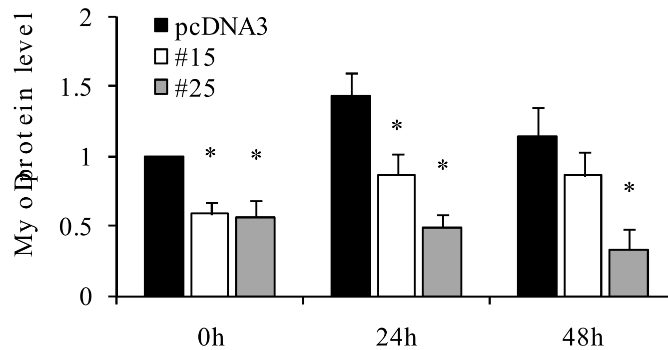

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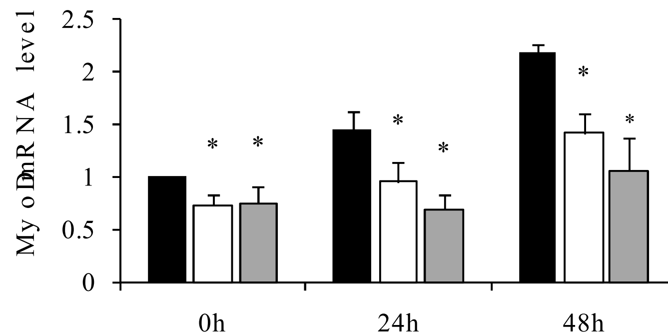
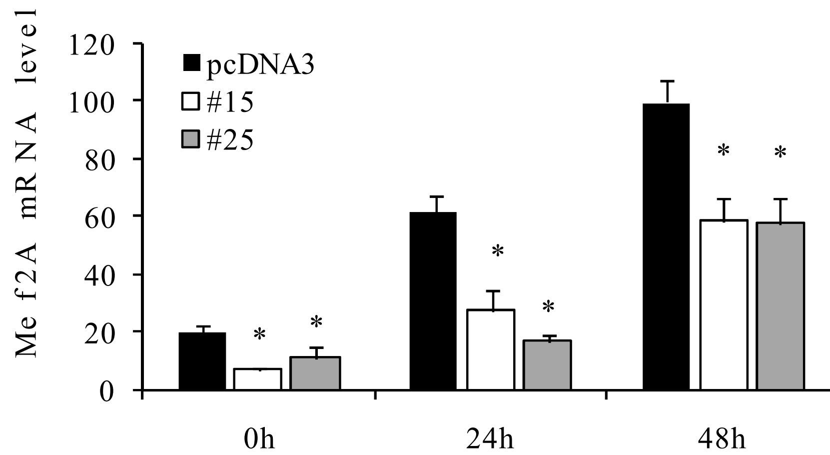


Figure 12: **Staufen1 over-expression decreases MyoD protein and mRNA levels.**

Clones #15 and #25 and the pcDNA3 control cells were left to proliferate in growth media (GM) until they reached 90% confluency, at which time differentiation was induced. Total proteins and RNA was extracted upon induction (0h), 24h and 48h after induction of differentiation. (A) MyoD and β-actin proteins were detected by Western blotting. Protein levels were normalized with β-actin. (B) MyoD protein levels were represented at 0h, 24h and 48h after induction of differentiation. Results are mean ± SE (n=3); *p<0.05. (C) *MyoD* mRNA levels were quantified by qRT-PCR relative to *cyclophilin B* mRNA levels. All the values are represented as fold changes over values derived from the pcDNA3 control, before induction of differentiation (0h). Results are mean ± SE (n=3, duplicates); *p<0.05.

A



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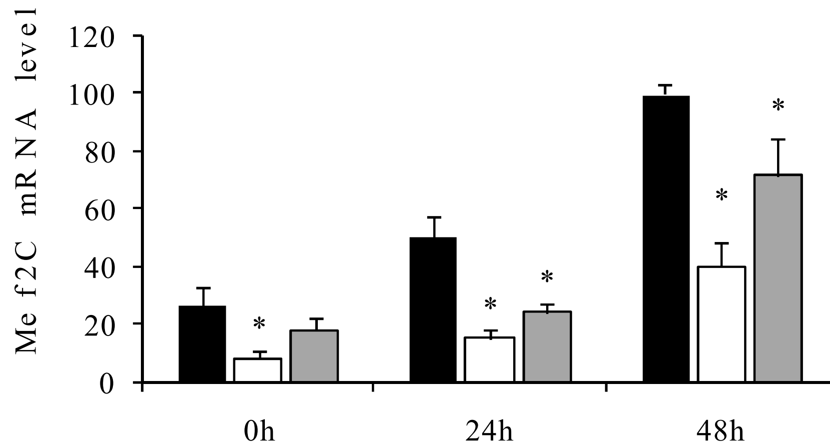

Figure 13: **Staufen1 over-expression decreases Mef2A and Mef2C mRNA expression during differentiation.**

Clones #15 and #25 and the pcDNA3 control cells were left to proliferate in growth media (GM) until they reached 90% confluency, at which time differentiation was induced. Total RNA was extracted upon induction (0h), 24h and 48h after induction. (A) *Mef2A* and (B) *Mef2C* mRNA levels were quantified by qRT-PCR relative to *cyclophilin B* mRNA levels and are represented as percentage fold changes over values derived from the pcDNA3 control, 48 hours after induction of differentiation. Results are means ± SE (n=3, duplicates); *p<0.05.

To determine if this decrease in mRNA expression was limited to Mef2A or if it extended to other members of the Mef2 family of transcription coactivators, we decided to look at Mef2C mRNA levels during differentiation. We found that like Mef2A, Mef2C mRNA levels were significantly decreased throughout differentiation. Before induction of differentiation, Mef2C mRNA levels were significantly lower only in clone #15 compared to those of in the pcDNA3 control ($p<0.05$) (Figure 13B). Once differentiation was induced, however, Mef2C mRNA levels were significantly lower in clone #15 and #25 compared to the levels found in the pcDNA3 control ($p<0.05$).

Taken together, these results suggest that both Mef2A and Mef2C are possible SMD targets and that the other members of this family could be SMD targets as well.

3.6. Over-expressing MyoD in the Staufen1 over-expressing stable cell lines rescues the delay in myogenin protein expression

In order to determine if the pathway involving MyoD is the main pathway affected by Staufen1 upregulation, we tried to rescue the defects in differentiation by over-expressing MyoD in the Staufen1 over-expressing clones #15 and #25. We transiently transfected clones #15, #25 and pcDNA3, seeded at the same density, with either an empty vector (CTL) or a vector containing the full MyoD cDNA sequence (MyoD). One day after transfection, differentiation was induced and cells were left to differentiate for 48 hours.

Total protein was extracted from these cells during proliferation (0h) and 24 hours and 48 hours after induction of differentiation. Western blots were performed to detect MyoD, myogenin expression and myogenin levels were quantified relative to β-actin. We did not

observe any difference in myogenin expression, throughout differentiation, between the pcDNA3 control cell line transfected with the empty plasmid and the pcDNA3 control cell line transfected with the plasmid containing MyoD (Figure 14B). We did however, observed a rescue in myogenin protein expression, 24 hours after induction of differentiation, for both clones #15 and #25 (Figure 14). As shown in Fig.14A, CTL transfected clones #15 and #25 express little or no myogenin after 24 hours of differentiation, as would be expected based on our previous results. On the other hand, these same clones transfected instead with the MyoD plasmid, expressed significantly more myogenin, 24 hours after induction of differentiation ($p<0.05$).

To better visualize the effects of the Staufen1 rescue on myotube morphology, we performed the same experiment described previously and differentiated the cells for 72h, at which time the cells were fixed and immunostained for MyCH protein, as described in materials and methods. Unlike our previous results, we observed a rescue in myotube size for clone #15 but not for clone #25 (Figure 14C), which, once again, could be explained by the fact that clone #25 expresses more Staufen1-HA than clone #15 (Figure 7).

3.7. Staufen1 over-expression affects cell cycle progression and arrest

It has previously been shown that c-myc over-expression inhibits C_2C_{12} cell differentiation and that this inhibition cannot be rescued by exogenous expression MyoD (Miner and Wold, 1991). It was also observed, by Weidensdorfer et al. (2009), that knocking down Staufen1 in U2OS cells led to a reduction in c-myc protein level without affecting mRNA levels and stability. An increase in c-myc protein levels in our stable cell lines over-expressing Staufen1 could not only explain the delay in differentiation that we have observed but it could also explain

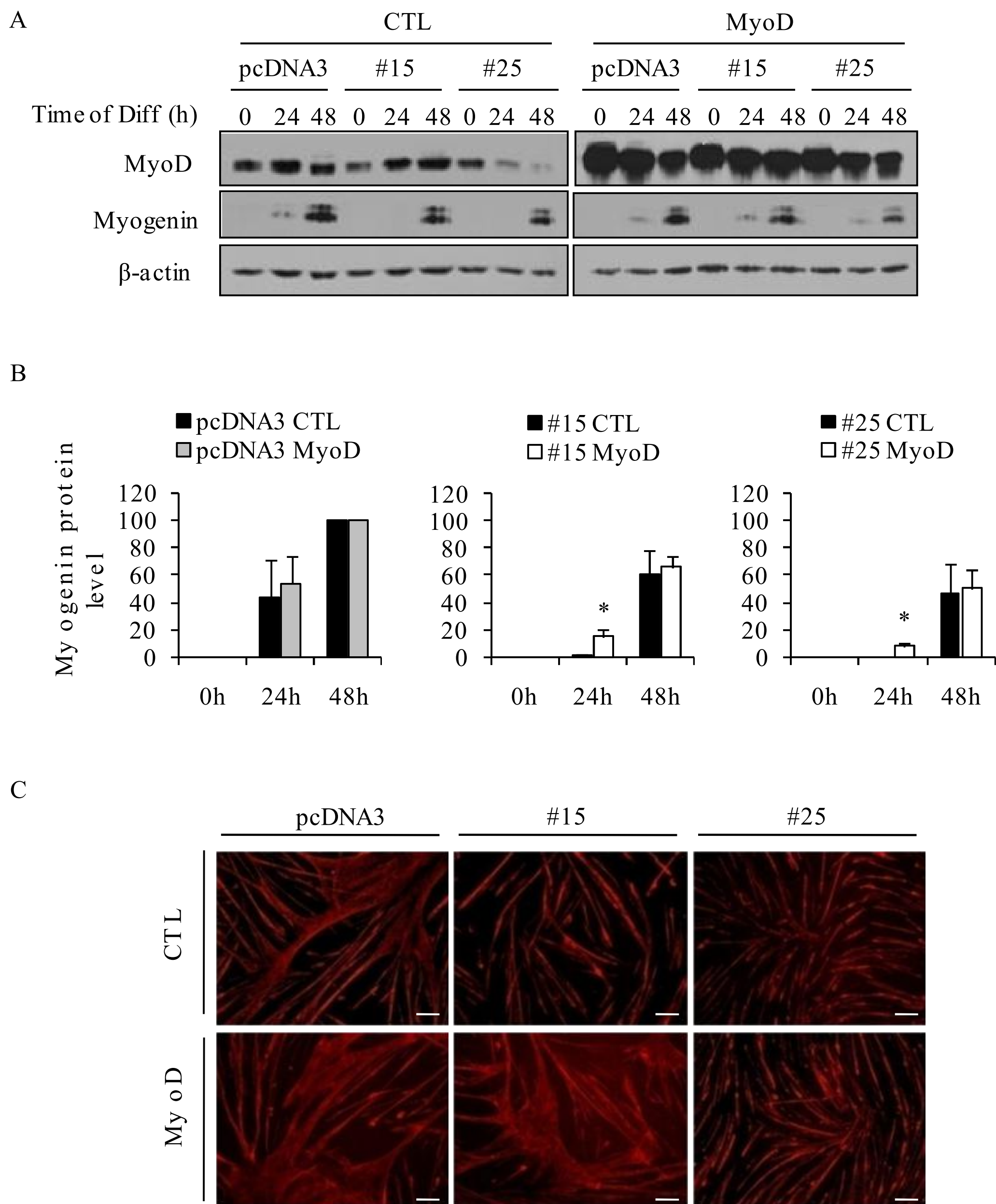

Figure 14: **Over-expressing MyoD in the Staufen1 over-expressing cells rescues the delay in myogenin protein expression.**

Clones #15 and #25 and the pcDNA3 control cells were transfected with either a MyoD expression vector (MyoD) or an empty vector (CTL). They were then left to proliferate in growth media (GM) until they reached 90% confluency, at which time differentiation was induced. Total protein was extracted upon induction (0h), 24h and 48h after induction. (A) MyoD, Myogenin and β-actin proteins were detected by Western blotting. (B) Myogenin protein levels were quantified relative to β-actin and are represented as percentage fold changes over values derived from the pcDNA3 CTL control, 48h after induction of differentiation. (C) MyHC was detected in CTL and MyoD transfected cells by immunohistochemistry, 72 hours after induction of differentiation. Pictures were taken at 2.5x magnification. Bar, 75µm. Results are mean ± SE (n=3); *p<0.05.

why the MyoD add-back experiment had no effect on the overall appearance of the myotubes formed by clone #25 even though myogenin protein expression was rescued.

We therefore decided to look at c-myc protein expression in our Staufen1 over-expressing stable clones and in C_2C_{12} cells that were transiently transfected with a pcDNA3 plasmid containing the Staufen1-HA construct. We found that c-myc protein expression was significantly increased in clones #15 and #25 compared to the pcDNA3 cells during proliferation ($p<0.05$), with clone #15 and #25 expressing 3 and 4 fold more c-myc than the pcDNA3 control (Figure 15B). This increase in c-myc protein levels was also observed upon transient transfection of C_2C_{12} cells with the Staufen1-HA construct compared to those transfected with an empty vector (Figure 16), confirming that Staufen1 was responsible for the increase in c-myc protein.

We then looked at c-myc protein levels during differentiation and found that upon induction of differentiation, c-myc protein levels decreased in our clones #15 and #25, to levels that were no longer significantly different than those in our pcDNA3 cells (Figure 15B). This decrease in c-myc levels during differentiation was also confirmed by transient transfection (Figure 16B). The fact that high c-myc protein expression was not maintained during differentiation could explain why we can only observe a delay and not complete inhibition of differentiation, in our cells over-expressing Staufen1. We therefore assume that differentiation would be completely inhibited and not delayed, in clones #15 and #25, if Staufen1 levels were maintained throughout differentiation at similar levels than during proliferation (Figure 7).

We then decided to look at c-myc mRNA levels by quantitative RT-PCR, during proliferation in our Staufen1 over-expressing clones to find out if this increase in c-myc protein corresponded to an increase in c-myc mRNA. We found that the c-myc mRNA levels in our

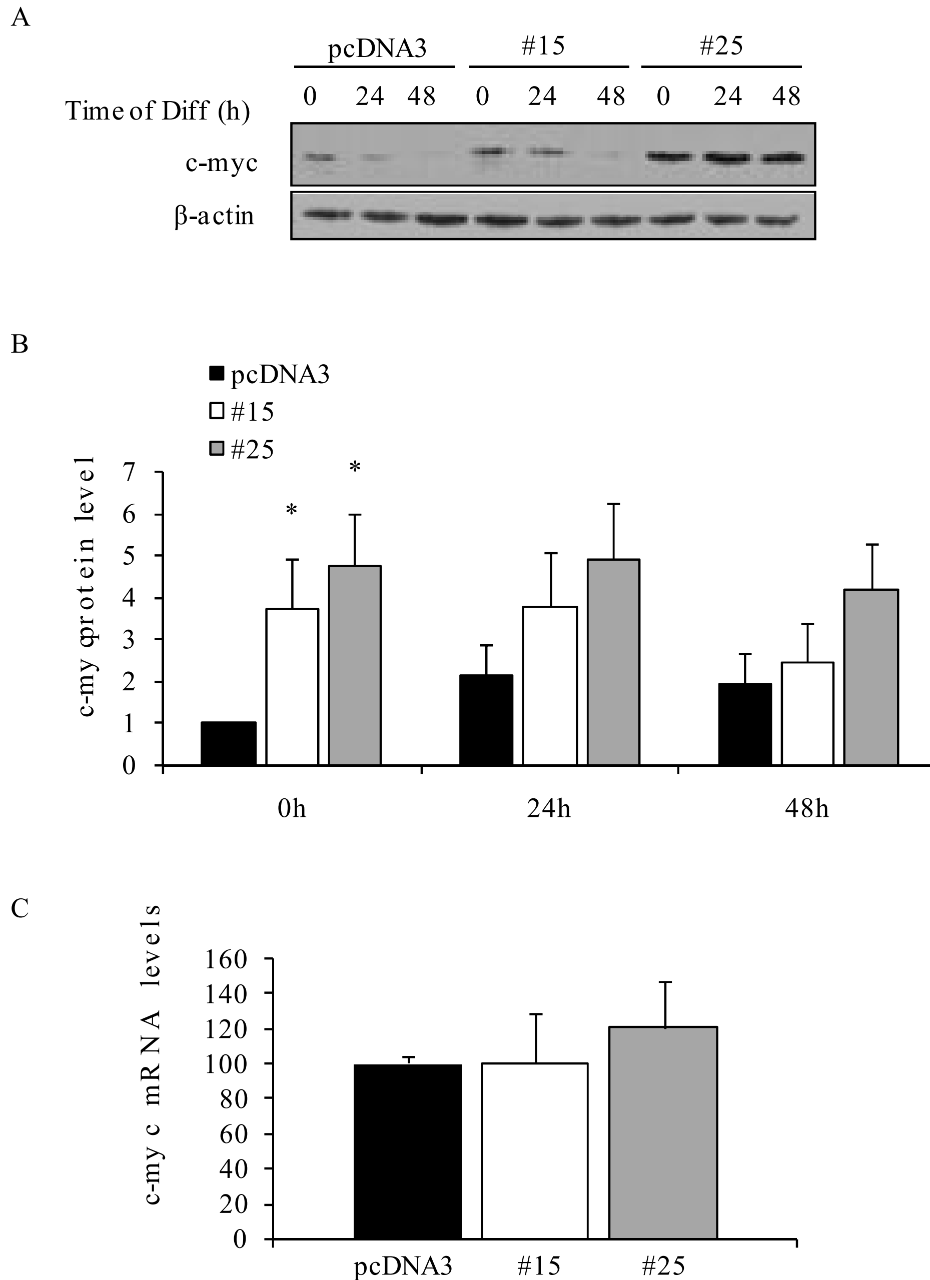

Figure 15: Staufen1 over-expression increases c-myc protein expression without increasing its transcription.

Clones #15 and #25 and the pcDNA3 control cells were seeded at the same density and cultured in growth media for 24 hours. Total protein and RNA was extracted during proliferation (0h), 24h and 48h after induction of differentiation. (A) c-myc and β-actin proteins were detected by Western blotting. (B) c-myc protein levels were quantified relative to β-actin and are represented as fold changes over values derived from the pcDNA3 control during proliferation (0h). Results are mean ± SE (n=4); *p<0.05. (C) *c-myc* mRNA levels were quantified by qRT-PCR relative to *cyclophilin B* mRNA levels and are represented as percentage fold changes over values derived from the pcDNA3 control during proliferation (0h). Results are means ± SE (n=4, duplicates).

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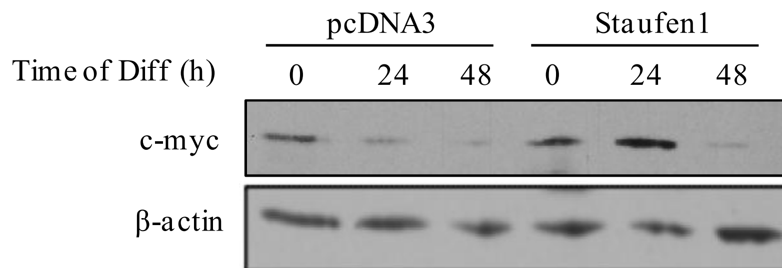

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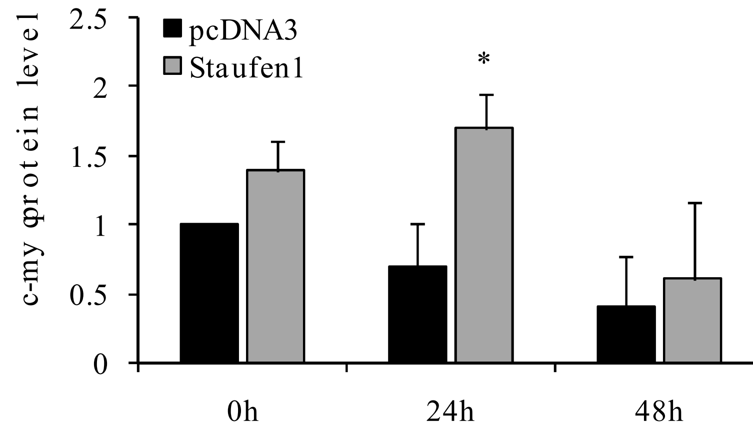


Figure 16: **Staufen1 over-expression, by transient transfection, increases c-myc protein expression during proliferation.**

C_2C_{12} cells were transiently transfected with a pcDNA3-Staufen1-HA construct and an empty pDNA3 plasmid as control. Total protein was extracted during proliferation (0h), 24h and 48h after induction of differentiation. (A) c-myc and β-actin proteins were detected by Western blotting. (B) c-myc protein levels were quantified relative to β-actin and are represented as fold changes over values derived from the pcDNA3 control during proliferation (0h). Results are mean ± SE (n=3); *$p<0.05$.

Staufen1 over-expressing cell lines were not significantly different than our pcDNA3 control cell lines (Figure 15C). This indicates to us that the increase in c-myc protein in the Staufen1 over-expressing clones is not due to an increase in c-myc transcription. It is possible that Staufen1 plays a role in the posttranscriptional regulation of the c-myc mRNA, in particular, by increasing c-myc translation through binding to its 5'UTR.

In order to test this hypothesis, we generated a c-myc 5'UTR/CAT construct with the long 5'UTR of c-myc. We then expressed this construct in our clones #15, #25 and pcDNA3 by transient transfection and performed CAT assays, as described in materials and methods. We found that there was no significant difference in CAT activity in our clones #15 and #25 compared to our pcDNA3 control, indicating that Staufen1 does not increase c-myc protein translation through the long 5'UTR (Figure 17). It has been established that there are three variants of the c-myc transcript, with two different 5'UTR: two transcripts with the long 5'UTR and one with a short 5'UTR (Trapnell et al, 2010). It is therefore possible that Staufen1 increases c-myc translation by binding to the short 5'UTR.

The proto-oncogene c-myc was first shown to play a significant role in cell proliferation by controlling cell cycle progression at different stages depending on the cell type (Dang et al, 1999). It was also shown that c-myc over-expression in HEL cells led to a significant increase in proliferation (Qi et al., 2007). For this reason we decided to perform several BrdU incorporation assays in order to observe the effects of Staufen1 on the proliferation of clones #15, #25 and the pcDNA3 control. Clones #15, #25 and pcDNA3 were cultured in GM and were exposed to BrdU

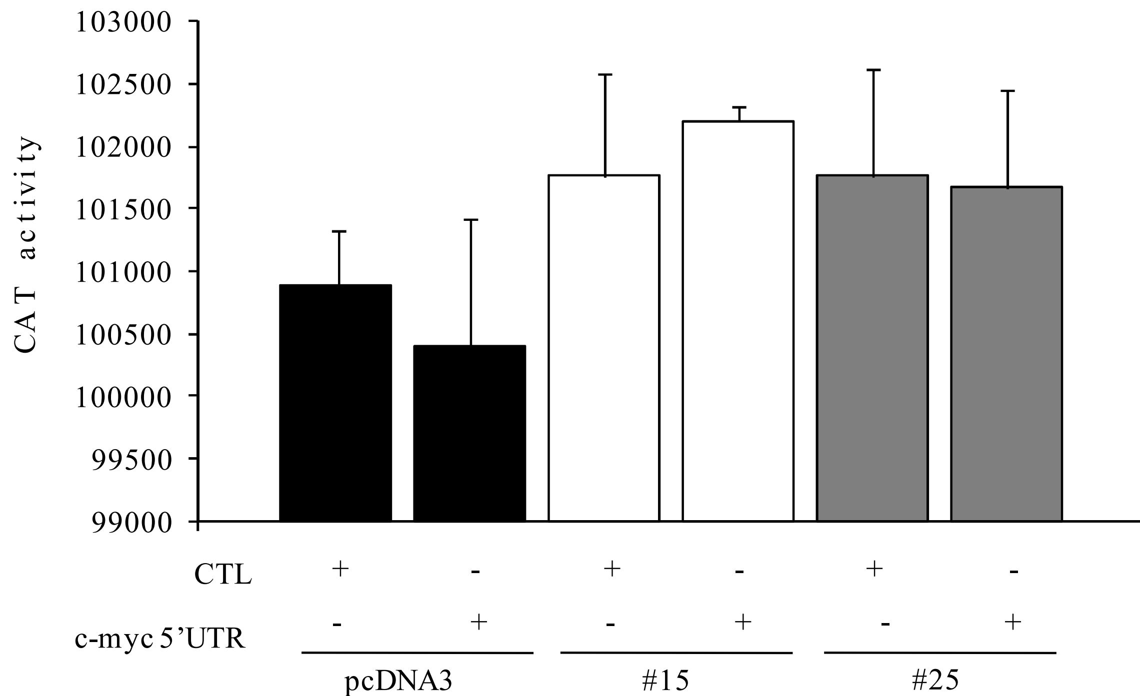
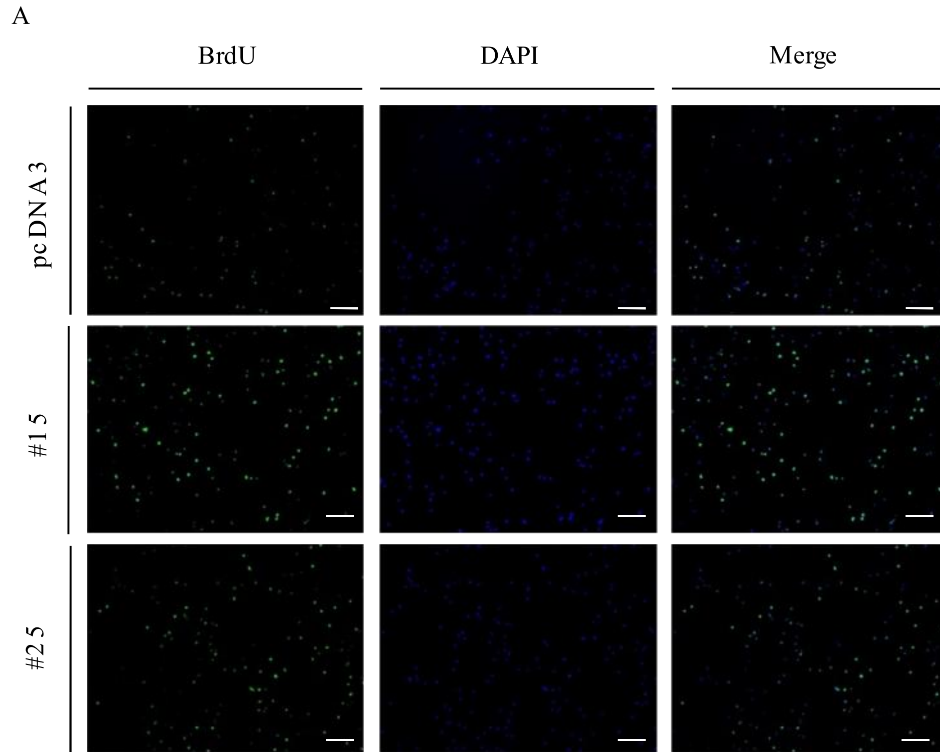


Figure 17: Staufen1 does not increase c-myc translation throught the long 5'UTR.

Clones #15, #25 and the pcDNA3 control cells transfected with either a c-myc long 5'UTR-CAT reporter construct or an empty CAT reporter plasmid. After which they were left to proliferate in growth media (GM) until they reached 90% confluency, at which time differentiation was induced by serum deprivation. The cells were lysed using passive lysis buffer and a CAT assay was performed to determine CAT activity. Results are means ± SE (n=1, triplicates).

for a period of 2 hours before fixation. Using fluorescence microscopy we were able to calculate the percentage of cells that had undergone proliferation during the 2 hour period of BrdU incorporation. As expected, we found that proliferation was slightly but significantly increased by 5% in the clones #15 and #25 compared to the pcDNA3 control ($p<0.05$) (Figure 18).

It is not entirely known exactly how c-myc inhibits differentiation, but it has been discovered that c-myc can directly inhibit the transcription of the cyclin-dependent kinase inhibitor p21, whose expression was shown to be necessary for differentiation to occur (Wu et al., 2003). We therefore looked at p21 mRNA levels by qRT-PCR and observed that after induction of differentiation p21 mRNA levels were significantly decreased in the clones #15 and #25 compared to the pcDNA3 cells ($p<0.05$) (Figure 19). This decrease in p21 mRNA would contribute to the delay in differentiation and the formation of smaller myotubes with fewer nuclei, by inhibiting cell cycle withdrawal which is a key step in the differentiation process.



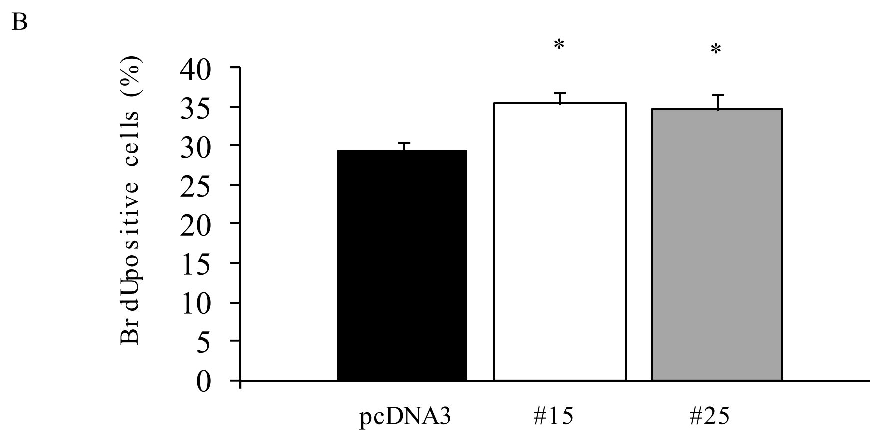


Figure 18: **Staufen1 over-expression increases myoblast proliferation.**

Clones #15 and #25 and the pcDNA3 control were seeded at the same density on glass coverslips and cultured in growth media for 24 hours. A BrdU proliferation assay, with a 2h BrdU pulse, was performed, (A) from pictures that were taken at 2.5X magnification (Bar, 75µm) and (B) the percentage of BrdU positive cells was calculated. Results are mean ± SE (n=4); *$p<0.05$.

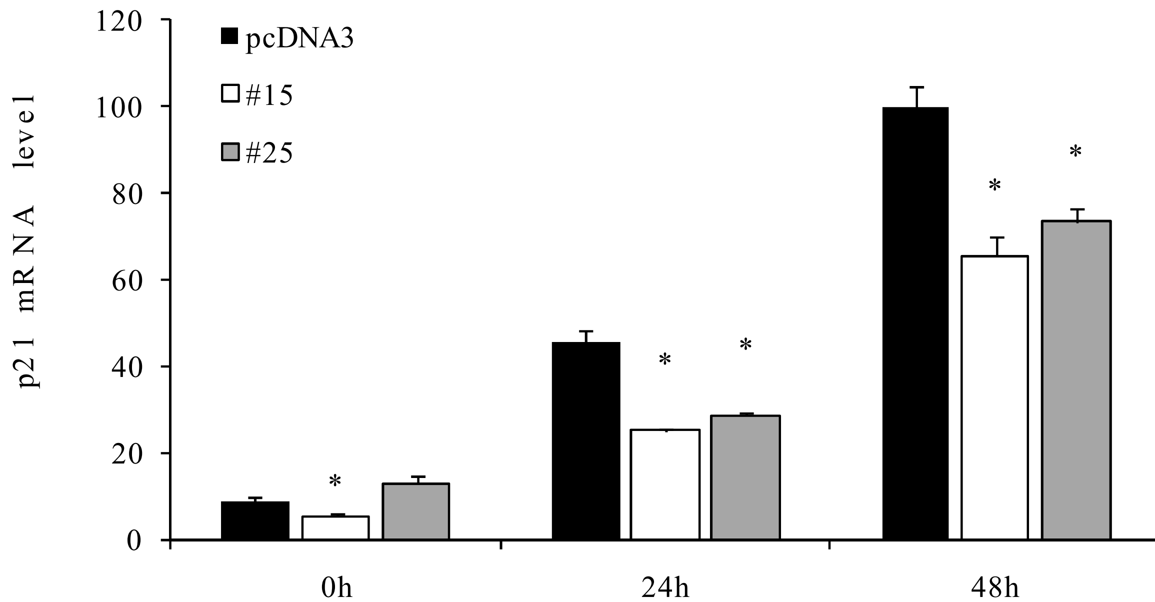


Figure 19: **Staufen1 over-expression decreases p21 mRNA expression during differentiation.**

Clones #15 and #25 and the pcDNA3 control cells were left to proliferate in growth media (GM) until they reached 90% confluency, at which time differentiation was induced. Total RNA weas extracted upon induction (0h), 24h and 48h after induction. *p21* mRNA levels were quantified by qRT-PCR relative to *cyclophilin B* mRNA levels and are represented as percentage fold changes over values derived from the pcDNA3 control, 48 hours after induction of differentiation. Results are means ± SE (n=3, duplicates); *p<0.05.

4. Discussion, preliminary result and future work

4.1. Discussion

4.1.1. Staufen1 over-expression delays C_2C_{12} differentiation

Even though Staufen1 has been extensively studied and has been implicated in many cellular processes including mRNA localization and transport, translation and decay, not much is known about its role in skeletal muscle. Therefore, to determine the role of Staufen1 in skeletal muscle differentiation and identify the mechanism or mechanisms by which it regulates this process, we generated C_2C_{12} stable cell lines that over-expressed Staufen1.

We initially observed that differentiation was delayed compared to a control cell line. To quantify this, we performed immunohistochemistry experiments on four day old myotubes which over-expressed Staufen1 and found that the DI, the FI and the average number of nuclei per myotube were significantly decreased compared to control myotubes.

We then looked at the expression of myogenin and MyHC, two myogenic genes that are normally upregulated during skeletal muscle differentiation and observed that Staufen1 over-expression decreased and delayed myogenin protein and mRNA expression as well as MyHC protein expression, during differentiation. As it was discussed earlier, activation of the myogenic program and expression of myogenic genes requires, among other things, the increased expression and transcriptional activity of MyoD and members of the Mef2 family of transcriptional regulators. We found that during proliferation and differentiation, Staufen1 over-expression significantly decreases MyoD protein and mRNA expression as well as Mef2A and Mef2C mRNA expression. The decreased expression of MyoD, Mef2A and Mef2C would lead

to a decrease in their transcription activity, which in turn would be responsible for the delay in myogenin and MyHC expression observed during differentiation, when Staufen1 is over-expressed in C_2C_{12} cells. It has been previously reported that, in cases where the pathway involving MyoD is the only one affected, over-expression of MyoD could rescue the delay in differentiation observed when MyoD levels were shown to be decreased (Bosnakovski et al., 2008). We therefore over-expressed MyoD in Staufen1 over-expressing cells and pcDNA3 control and found that MyoD over-expression only partially rescued the differentiation defects observed in the cells over-expressing Staufen1. We found that the delay in myogenin protein expression was rescued in our cells over-expressing Staufen1, however, we only rescued the defects in myotube formation in one of two stable cell lines tested. We believe that the extent of the rescue, when MyoD is over-expressed, depends on Staufen1 expression levels. These results indicate that the pathway involving MyoD is not the only one affected by Staufen1 over-expression.

As it was mentioned earlier, in order for terminal differentiation and myoblast fusion to occur, the myoblasts must withdraw from the cell cycle. This process requires the expression of p21, which is regulated by both c-myc and MyoD. We initially found that Staufen1 over-expression increases c-myc protein expression during proliferation but does not affect c-myc mRNA levels. This led us to believe that Staufen1 increases c-myc translation by binding to its 5'UTR. We tested this hypothesis using a reporter construct containing one of the two possible 5'UTRs of c-myc and determined that Staufen1 did not increase c-myc translation through the long 5'UTR. This, however, does not exclude the possibility that Staufen1 increases c-myc translation through the short 5'UTR. To confirm that the increase in c-myc expression, observed when Staufen1 is over-expressed, affected cell cycle withdraw, we looked at myoblast

proliferation and p21 expression and found that proliferation was slightly but significantly increased before induction of differentiation and that p21 mRNA expression was significantly decreased during differentiation. The upregulation of c-myc protein expression, when Staufen1 is over-expressed, is therefore important in explaining the delay observed during skeletal muscle differentiation, since it decreases p21 transcription and promotes cell cycle progression.

All of these results point to the fact that Staufen1 negatively regulates skeletal muscle differentiation, through the posttranscriptional regulation of one or more transcripts, which we believe could be c-myc, Mef2A and Mef2C.

4.1.2. Staufen1 is a negative regulator of skeletal muscle differentiation

For more than a quarter of a century Staufen1 has been studied in the context of embryonic development in *Drosophila* and neurogenesis. It is only recently, starting with the work done by the group of Dr. Bernard Jasmin, that the role of Staufen1 in muscle and myogenesis has been investigated (Bélanger et al., 2003). Two independent groups, the first led by Dr.Lynne E. Maquat and the other led by Dr.Kenji Irie, later studied the role of Staufen1 during skeletal muscle differentiation and found opposite results: the first found that Staufen1 is a positive regulator while the second found that it is a negative regulator of muscle differentiation (Gong et al.2008 and Yamaguchi et al. 2008).

The group led by Dr.Lynne E. Maquat came to the conclusion that Staufen1 is a positive regulator of skeletal muscle differentiation through its work on Saufen1 in SMD. As it was mentioned previously, they found that SMD targets c-jun and Pax3, known to inhibit differentiation, were decreased during skeletal muscle differentiation, while the NMD target

myogenin, know to promote differentiation, was increased (Gong et al, 2009). Since both SMD and NMD require Upf1 to induce mRNA decay, they hypothesized that SMD promotes myogenesis through competition with NMD for access to Upf1. While the results published in these papers are important and will help to identify possible SMD targets during differentiation, a few minor issues can were identified and should be addressed. For example, unlike in the Kim et al. (2007) paper, which showed that Staufen1 levels increased during differentiation, the Gong et al. (2009) paper found that Staufen1 protein levels were decreased two days after induction of differentiation compared to proliferating myoblasts. This type of problem seems to occur relatively often in the field of molecular biology and is most likely cause by the fact that two different antibodies were used to detect Staufen1. It therefore still remains to be determined whether endogenous Staufen1 increases or decreases during C_2C_{12}differentiation. Another example can be found in the Gong et al. (2009) paper in which they state that Staufen1 promotes C_2C_{12} differentiation by decreasing the mRNA levels of PAX3, a known inhibitor of differentiation. Many different groups have looked at the endogenous expression of PAX3 in C_2C_{12} and were not able detect any mRNA or protein expression, from which they concluded that C_2C_{12} do not express PAX3 (Kuang et al.2006, Collins et al.2009, Gherzi et al., 2010). There are a few possible explanations for this, such as possible genomic DNA contamination of their RT-PCRs or the fact that the primers used to amplify PAX3 somehow were amplifying another transcript.

The group led by Dr.Kenji Irie, on the other hand, found that Staufen1 was a negative regulator of skeletal myogenesis. They knocked-down endogenous Staufen1 expression in C_2C_{12} cells and observed that differentiation occurred spontaneously without induction and was accompanied by an increase in myoglobin and myogenin protein expression (Yamaguchi et al.,

2007). They went even further by saying that SMD is not involved in skeletal muscle differentiation, since they found that knocking down Upf1 did not induce the expression of myogenin without induction, and that expression of Staufen1^{i} (a Staufen1 isoform which lacks RNA-binding activity due to the insertion of six amino acids within the dsRBD3) had the same effect as the expression of wild type Staufen1. Even though these experiments could indicate that that SMD is not involved in the differentiation process, we believe that it would have been more conclusive if they had knocked down endogenous Staufen1 and expressed a Staufen1 construct which lacked the dsRBD4 and the TBD, which are the domains that have been shown to interact with Upf1 during SMD (Kim et al., 2005). Since, knocking down Upf1 would not only inhibit SMD but would also inhibit NMD and expressing Staufen1^{i} does not completely inhibit Staufen1 RNA-binding activity since the RNA-binding activity of the dsRBD4 still remains (Wickham et al., 1999). Interestingly, in order to replicate these results, both Dr.Lynne E. Maquats group and our group endeavored to generate stable C_2C_{12}cells lines where Staufen1 was knocked down by siRNA, however we were both unsuccessful in doing so.

Even though research on the role of Staufen1 in skeletal muscle differentiation has already been done, opposing conclusions were obtained. A detailed analysis of these papers has led us to believe that further research on the matter was necessary. Taking into account the work that has already been done; we decided that the best approach to determine the role of Staufen1 in skeletal muscle differentiation was to generate C_2C_{12} stable cell lines over-expressing Staufen1. Based on the results obtained from these stable cell lines, we determined that Staufen1 is a negative regulator of skeletal muscle differentiation. We have not yet been able to determine the precise mechanism by which Staufen1 regulates myogenesis but we do not exclude the possibility that SMD is involved and we have identified two potential SMD targets: Mef2A and

Mef2C. We also bring forward the idea that the posttranscriptional regulation of c-myc, by Staufen1, is important in mediating its effects on skeletal muscle differentiation.

4.1.3. Staufen1 and posttranscriptional regulation during C_2C_{12} differentiation

As it was mentioned previously, the posttranscriptional regulation of specific transcripts is an important aspect of skeletal muscle differentiation. We illustrated this with two examples, the RNA-binding proteins HuR and CUGBP1, which were both shown to play important roles in myogenesis, through the stabilization and increased transcription of myogenic factors. Since Staufen1 has been implicated in multiple different posttranscriptional processes, such as mRNA transport, translation and decay, and since we have found that its over-expressing in C_2C_{12} cells inhibited proper differentiation, we believe that, like HuR and CUGBP1, Staufen1 plays an important role in skeletal muscle differentiation through the posttranscriptional regulation of specific transcripts. We have been able to identify three possible targets of Staufen1 that would explain the delay observed in our Staufen1 over-expressing cell lines: c-myc, Mef2A and Mef2C.

We know that Staufen1 plays a role in modulating c-myc protein abundance given that Weidensdorfer et al. (2009) found that c-myc protein levels were significantly reduced upon Staufen1 knockdown in U2OS cells and that c-myc mRNA stability and expression remained unaffected. In agreement with these finds, we observed that upon Staufen1 over-expression in C_2C_{12} cells, c-myc protein levels were significantly increased, while c-myc mRNA levels remained unchanged. In view of the fact that Staufen1 has been reported to have the ability to increase translation of specific mRNAs with structured 5'UTR (Dugré-Brisson et al, 2005), it is

possible that the changes in c-myc protein expression could be attributed to Staufen1 interacting directly with its 5'UTR and increasing its translation. We tested this hypothesis and found that Staufen1 over-expression did not increase c-myc protein translation through its long 5'UTR. Nevertheless, it is still possible that Staufen1 over-expression increased the translation of the c-myc transcript possessing the short 5'UTR, which was shown to be preferentially expressed during differentiation (Trapnell et al., 2010).

We have also been able to identify two other potential Staufen1 targets that are involved in the myogenic program: Mef2A and Mef2C. It has been shown previously, by immunopurification and subsequent microarray analysis, that Mef2A mRNA can be found in Staufen1^{55}-HA containing ribonucleoprotein (mRNP) complexes (Furic et al., 2008). Based on the experimental protocol described, it would indicate that Staufen1^{55}-HA has the ability to bind Mef2A mRNA, making it a possible SMD target. If Mef2A is an SMD target then we would assume that its mRNA expression would decrease if Staufen1 expression was increased. This could explain the differentiation defects in our Staufen1 over-expressing cell lines, since, as it has been mentioned previously, the expression of members of the Mef2 family of regulatory factors is necessary for the normal progression of skeletal muscle differentiation. We therefore looked at Mef2A and Mef2C mRNA levels before and during differentiation and showed that, in both cases, it was significantly reduced when Staufen1 was over-expressed.

It is important to point out, however, that even if Mef2A and Mef2C are determined to be SMD targets they could not explain the increase in c-myc protein that we have observed. This leads us to believe that they are not the only Staufen1 target affected during differentiation. It is likely that there are still other transcripts that are regulated by Staufen1 during differentiation. Since direct binding of Staufen1 to a transcript is necessary to increase their transcription or

cause decay, the information that can be found in the microarray data of the Furic et al. (2009) and Kim et al.(2007) are invaluable in the search for possible targets. After having looked through them, we were able to identify multiple different transcripts that play a role in myogenesis, that are bound by Staufen1 and whose expression changes upon Staufen1 downregulation. In this study, we focused our attention on the most obvious ones but others should still be tested.

4.2. Preliminary result

Since Mef2A and Mef2C could not be SMD targets that could explain all the changes that we observed in C_2C_{12} over-expressing Staufen1, during differentiation, we continued our search for more possible SMD targets. We concentrated mostly on targets that would be involved in one of the two main pathways that lead to muscle differentiation: the p38/MAPK pathway and the PI3K-AKT-p70S6K pathway. We found that the p35 transcript, necessary for activation of differentiation through the PI3K-AKT-p70S6K pathway, was shown to be upregulated in human cells depleted of Staufen1 and shown to bind Staufen1-HA (Kim et al., 2007), making it an ideal SMD target. Depletion of p35 would, theoretically, decrease CDK5 activity and therefore not only inhibit differentiation (Lazaro et al., 1997, Sarker et al., 2004) but would also lead to an increase in c-myc protein stability through the increased activation of the MEK1-ERK1/2 pathway (Sharma et al., 2002, Yeh et al., 2004). We therefore looked at p35 mRNA expression in Staufen1 over-expressing C_2C_{12} cells before and after induction of differentiation and found that levels were significantly lower than in control cells (Figure 20). Even though we saw results

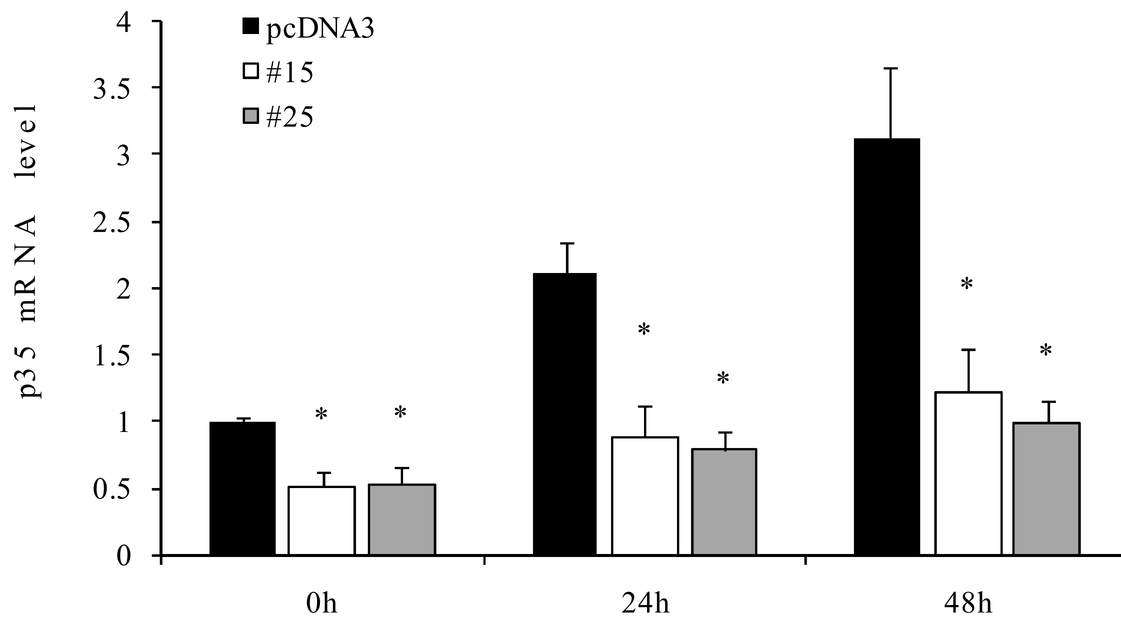


Figure 20: **Staufen1 over-expression decreases p35 mRNA expression during differentiation.**

Clones #15 and #25 and the pcDNA3 control cells were left to proliferate in growth media (GM) until they reached 90% confluency, at which time differentiation was induced by serum deprivation. Total RNAs were extracted upon induction (0h), 24h and 48h after induction. *p35* mRNA levels were quantified by qRT-PCR relative to *cyclophilin B* mRNA levels and are represented as fold changes over values derived from the pcDNA3 control, before induction of differentiation (0h). Results are means ± SE (n=3, duplicates); *$p<0.005$.

that are consistent with p35 being an SMD target, it is still necessary to investigate the matter further.

4.3. Future work

In this study we have shown that Staufen1 is a negative regulator of skeletal muscle differentiation. We have identified four possible targets of Staufen1 that have been shown to play an important role skeletal myogenesis: c-myc, Mef2A, Mef2C and p35. However, more experiments are required to confirm that they are posttranscriptionally regulated by Staufen1 during skeletal muscle differentiation.

We have found that c-myc protein expression was increased when Staufen1 was over-expressed but that its mRNA expression remained unchanged. This led us to believe that Staufen1 increased c-myc translation by binding to its 5'UTR. We tested this hypothesis by reporter assay, using a reporter construct containing the long 5'UTR of c-myc, and found that Staufen1 did not increase c-myc translation through the long 5'UTR. It has been established that three c-myc transcripts are produced by the c-myc gene and that they possess two different 5'UTR: a short and a long 5'UTR. In order to determine if Staufen1 increases c-myc translation, it is still necessary to test the short 5'UTR, following the same protocol that was described for the long 5'UTR. If we find that reporter expression is increased when Staufen1 is over-expressed we will then have to perform RNA immunoprecipitation experiments and perform band shift assays to confirm that Staufen1 binds the c-myc transcript through the 5'UTR.

We have also identified three transcripts that are most likely SMD targets since they have been shown to bind Staufen1[55] and in the case of p35 to be upregulated upon Staufen1 depletion

(Furic et al., 2008, Kim et al., 2007) . In this study we looked, by qRT-PCR, at the mRNA expression of these three transcripts and have found that they were all significantly decreased when Staufen1 was over-expressed, both during proliferation and differentiation. In order to confirm that they are SMD targets a few more experiments are required. We will first have to test whether Staufen1 regulates these transcripts through their 3'UTRs, by generating reporter constructs containing the 3'UTR of Mef2A, Mef2C and p35, and perform reporter assays, as described in material and methods. As it was mentioned previously we should then confirm that Staufen1 binds the 3'UTR of these transcripts by performing band shift assays.

To confirm the role of SMD in skeletal muscle differentiation, it would be interesting to generate C_2C_{12} stable cell lines over-expressing a mutant Staufen1 lacking the dsRBD4 and the tubulin binding domain, the two domains that were shown to be required for binding to Upf1 (Kim et al., 2005). We would then investigate its effects on the differentiation process and compare the results obtained to those obtained from this study. We could then confirm that Mef2A, Mef2C and p35 are SMD targets and also uncover other possible SMD targets that could mediate the effects of Staufen1 on skeletal muscle differentiation.

We should also further examine the possibility that p35 is the SMD target that is responsible for both the decreased expression of myogenic genes and the increase in c-myc protein expression, when Staufen1 is over-expressed. Since p35 is relatively far upstream in the PI3K-AKT-p70S6K, it is not only important to prove that it is an SMD target, it is also necessary to show that p35/CDK5 activity is reduced in our Staufen1 over-expressing stable cells lines and that the reduction if p35/CDK5 activity affects both the p53 and the MEK/ERK pathways.

4.4. Proposed model

Based on our findings we believe that Staufen1 negatively regulates differentiation through two posttranscriptional regulation mechanisms: mRNA decay and increased translation. We therefore propose this model to show the possible consequences of the posttranscriptional regulation of c-myc, Mef2A, Mef2C and p35, by Staufen1, on skeletal muscle differentiation when Staufen1 is over-expressed (Figure 21).

If p35 is an SMD target, its expression would be decreased when Staufen1 is upregulated. This would decrease CDK5 activation, which would, through the p35/CDK5-p53-Rb pathway lead to the decreased expression and activation of MyoD, Mef2A and Mef2C and also increase the protein stability of c-myc. Consequently expression of myogenin, p21 and MyHC would be decreased and differentiation and fusion would be affected.

It is also likely that Staufen1 regulates not only p35 but also regulates Mef2A, Mef2C and c-myc and that it is through the combined regulation of all these transcripts and others that Staufen1 mediates its effects on skeletal muscle differentiation.

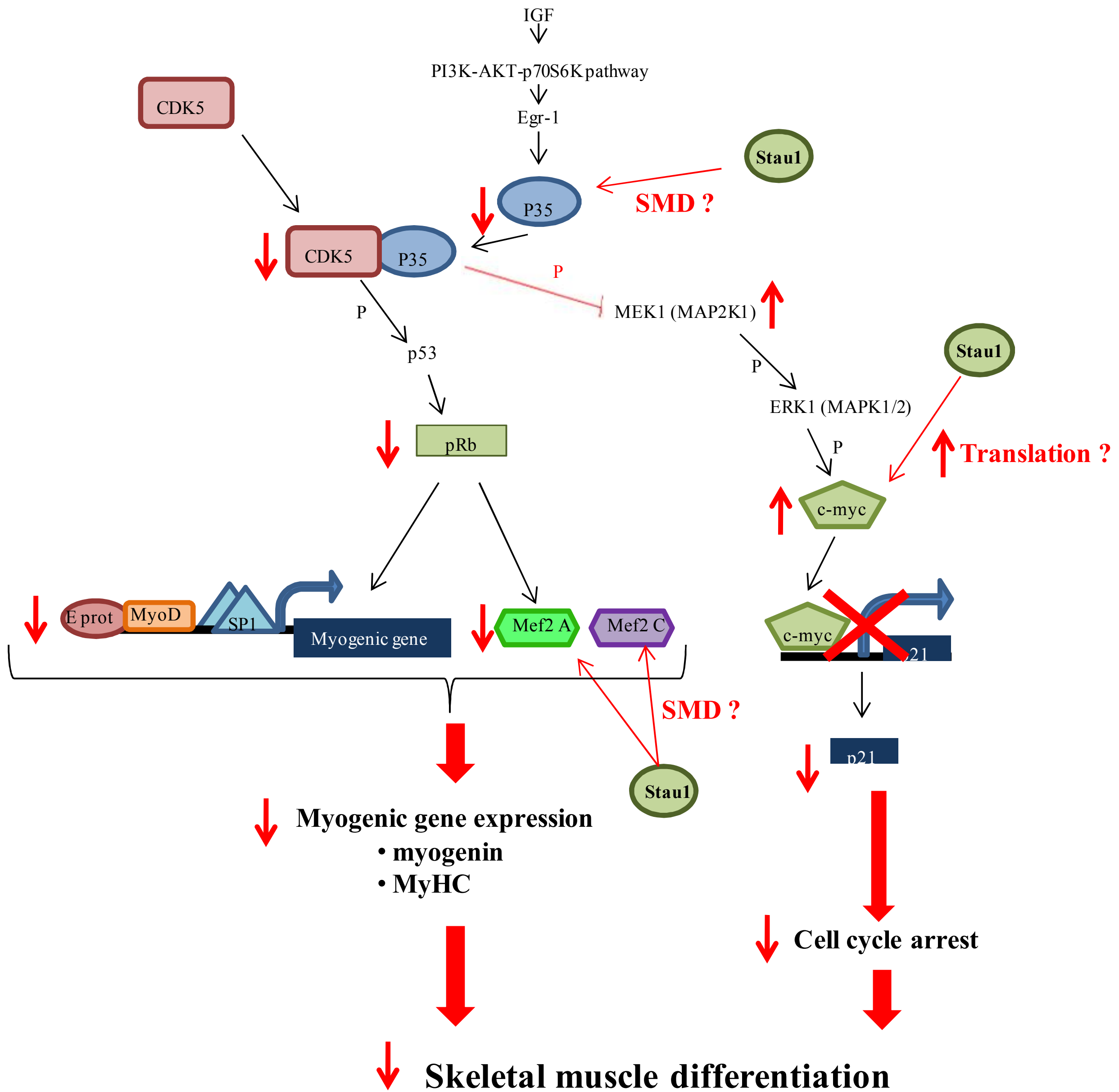


Figure 21: **Proposed Model.**

Our study has found that Staufen1 is a negative regulator of skeletal muscle differentiation. We propose that Staufen1 mediates its effects on this process through the posttranscriptional regulation of four potential targets: c-myc, Mef2A, Mef2C and p35. Staufen1, through SMD, could decrease p35 expression, which would reduce binding (B) of p35 to CDK5 and consequently decreasing p35/CDK5 activity. This would prevent differentiation by inhibiting p53 and MEK1/EK1 phosphorylation (P) which would ultimately inhibit the expression of myogenic genes and prevent cell cycle withdrawal. The inhibition of skeletal muscle differentiation when Staufen1 is upregulated could also be caused by a decrease Mef2A and Mef2C expression, though SMD, as well as an increase in c-myc expression, through Staufen1 binding its short 5'UTR and increasing its translation.

4.5. Conclusion

The goal of this study was to determine the role of Staufen1 during myogenic differentiation, identify potential targets and infer a mechanism through which it would mediate its effects. We therefore generated C_2C_{12} stable cell lines that over-expressed Staufen1. We first observed that Staufen1 over-expression delayed differentiation and led to the formation of smaller myotubes. We determined by immunohistochemistry that the DI, the FI and the average number of nuclei per myotube was significantly decreased in the Staufen1 over-expressing stable cell lines. To better understand the effects of Staufen1 over-expression on the differentiation process, we looked at the expression of the myogenic regulators MyoD, Mef2A and Mef2C and found that their expression was significantly decreased before and after induction of differentiation. To determine if the main pathway affected by Staufen1 involved MyoD, we performed a MyoD add-back experiment and observed that the delay in myogenin protein expression was rescued but that the defects in myotube size were not completely rescued. This indicated to us that another pathway, independent of MyoD, was also affected by Staufen1. Interestingly, we found that when Staufen1 was over-expressed, c-myc protein expression was significantly increased while its transcript levels remained unchanged. The increase in c-myc observed would explain the lack of complete rescue when MyoD is added back. Consistent with these previous results, we found that Staufen1 over-expression decreases and delays the expression of the early and late myogenic markers, myogenin and MyHC, as well as the CDK inhibitor p21, which taken together, would lead to decreased differentiation, cell cycle withdrawal and fusion. From these results we concluded that Staufen1 is a negative regulator of skeletal muscle differentiation and we propose that its role in this process is mediated through the posttranscriptional regulation of c-myc, Mef2A, Mef2C and p35.

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