The role of nTRIP6 in adult myogenesis

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I Zusammenfassung

Die Regeneration der Sekelettmuskulatur erfolgt durch ansässige adulte Stammzellen, die sogenannten Satellitenzellen. Sobald ein Schaden an den Muskeln auftritt werden diese Zellen aktiviert und fangen als Myoblasten an zu proliferieren. Diese Myoblasten verlassen dann den Zellzyklus, und differenzieren zu Myozyten, welche dann miteinander fusionieren, um neue Myofasern zu bilden. Dieser Prozess wird adulte Myogenese genannt und wird durch ein Netzwerk von Transkriptionsfaktoren kontrolliert. Einer dieser Transkriptionsfaktoren ist MEF2C, welcher die Expression solcher Gene begünstigt, die an der späten Differenzierung und Fusion beteiligt sind. Allerdings ist MEF2C bereits während der späten Proliferation exprimiert. Aus diesem Grund muss die transkriptionelle Aktivität von MEF2C kontrolliert werden. Vorangegangene Arbeit dieses Labors hat nTRIP6, die nukläre Isoform des LIM Domänen Proteins TRIP6 identifiziert, welches als Koaktivator für verschiedene Transkriptionsfaktoren agiert. Basierend auf der beschriebenen Interaktion zwischen MEF2C und TRIP6, stelle ich die Hypothese auf, dass nTRIP6 als transkriptioneller Koregulator für MEF2C agiert. In proliferierenden Myoblasten interagiert nTRIP6 mit MEF2C und wurde zusammen mit MEF2C zu den regulativen Abschnitten von MEF2C Zielgenen rekrutiert, was zu der Repression von MEF2C führt. Deshalb agiert nTRIP6 als transkriptioneller Korepressor für MEF2C. Interessanter weise, ist die Expression von nTRIP6 während der späten Proliferation und der beginnenden Differenzierung, vorrübergehend gesteigert. Dies ist der kritische Moment an welchem die Aktivität von MEF2C reprimiert werden muss. Sobald die Funktion von nTRIP6 blockiert wird, steigert sich die Expression von Markern der späten Differenzierung, in proliferierenden Myoblasten. Dieser Vorgang wurde von einem Defekt in der Fusion zu einem späteren Zeitpunkt begleitet. Deshalb scheint es so, als würde die zeitliche Regulation der Expression von nTRIP6 die vorzeitige Differenzierung verhindern, um eine korrekte Kontrolle über die Fusion zu gewährleisten. Zusätzlich zeigen sich regenerierende Muskelfasern, in einem Modell für Muskelregeneration in der Maus, bei dem das *trip6* Gen in Satellitenzellen ausgeschaltet wurde, eine kleinere Größe der Muskelfasern und mehr unfusionierte myogene Zellen, im Vergleich zu den Kontrolltieren. Zusammengefasst habe ich eine neue molekulare Funktion von nTRIP6 als Korepressor von MEF2C gefunden, sowie eine neue Rolle für nTRIP6 als Modulator der Dynamik der adulten Myogenese.

II Abstract

Skeletal muscle regeneration is carried out by resident adult stem cells, the so-called satellite cells. Upon muscle damage, these cells get activated and start to proliferate as myoblasts. Myoblasts then exit the cell cycle, differentiate into myocytes which then fuse to form new myofibres. This process, referred to as adult myogenesis, is regulated by a network of transcription factors. One such transcription factor is MEF2C, which drives the expression of genes involved in late differentiation and fusion. However, MEF2C is already expressed during late proliferation. Thus, its transcriptional activity must be controlled. Previous work of the lab has identified nTRIP6, the nuclear isoform of the LIM domain protein TRIP6, as a co-activator for several transcription factors. Based on a described interaction between MEF2C and TRIP6, I hypothesized that nTRIP6 acts as a transcriptional co-regulator for MEF2C. In proliferating myoblasts, nTRIP6 interacted with MEF2C and was recruited together with MEF2C to the regulatory regions of MEF2C target genes, resulting in the repression of MEF2C activity. Thus, nTRIP6 acts as a transcriptional co-repressor for MEF2C. Interestingly, nTRIP6 expression was transiently increased during late proliferation and early differentiation of myoblasts, the critical period when MEF2C activity has to be repressed. When nTRIP6 function was blocked, the expression of late differentiation markers was increased in proliferating and early differentiating myoblasts. This was accompanied by a defect in the fusion of myocytes at later time points. Thus, the temporal regulation of nTRIP6 expression appears to prevent premature differentiation, which is necessary for a proper control of fusion. In addition, in a mouse model of muscle regeneration, when the *trip6* gene was knocked out in satellite cells the regenerated muscle showed a smaller myofibre size and more unfused myogenic cells than in the control animals, showing the *in vivo* relevance of my findings. In conclusion, I uncovered a novel molecular function for nTRIP6 as a co-repressor for MEF2C, as well as a novel role for nTRIP6 as a modulator of the dynamics of adult myogenesis.

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V Abbreviations

AP-1	Activator protein 1
bHLH	Basic helix-loop-helix
BiFC	Bimolecular fluorescence complementation
ER	Estrogen receptor
GR	Glucocorticoid receptor
HA	Hemagglutinin
HDAC4/5	Histone deacetylase 4/ 5
KO	Knockout
Luc	Luciferase
mCherry	Cherry- red fluorescent protein
MEF2C	Myocyte enhancer factor 2
MhC	Myosin heavy chain
MRF	Myogenic regulatory factor
MYOD	Myoblast determination protein
MYF5	Myogenic factor 5
MYH3	Embryonic myosin heavy chain
NES	Nuclear export signal
NLS	Nuclear localization sequence
NF-κB	Nuclear factor κB
nTRIP6	Nuclear isoform of TRIP6
PBS	Phosphate buffered saline
S.D.	Standard deviation
TRIP6	Thyroid hormone receptor interacting protein 6
Tris	Tris(hydroxymethyl)-aminomethane
VC	C- terminal part of Venus fluorescent protein
VN	N- terminal part of Venus fluorescent protein
WT	Wildtype

1. Introduction

1.1 Skeletal muscle regeneration

Muscle tissue contributes to approximately 45% of total body mass and represents the largest organ in the human body (Goodpaster et al., 2000). Skeletal muscles are responsible for locomotion and force generation and form a huge metabolic organ. Skeletal muscles comprise muscle fibre bundles that include individual myofibers. Myofibers are multinucleated syncytial cells that are formed by the fusion of progenitor cells. Within each of these myofibers many myofibrils are located that form a repetitive array of a specialized contractile structure, the sarcomere. The sarcomere contains two major types of filaments: thick filaments composed of myosin II and thin filaments containing actin and other associated proteins. When the muscle contracts, changes in the interaction between myosin and the actin permits contraction. Individual myofibers within the muscle are exposed to intrinsic tensile force generated when the muscle contracts. This force can make the myofibres rupture even during daily life. Thus, skeletal muscle is constantly damaged and has to be repaired or regenerated if the damage has led to cell death.

Muscle regeneration can be divided into three phases. In the first phase, the destruction phase, the damaged myofibres become necrotized and the damaged part is filled by a haematoma. In the next phase (repair phase), phagocytosis of the necrotized tissue by infiltrated macrophages takes place. In the last phase (remodelling phase), newly formed muscle tissue is generated in a process called adult myogenesis (Schiaffino and Partridge, 2008). In skeletal muscle, the regeneration process is driven by adult muscle stem cells, the so-called satellite cells. The name satellite cells comes from their location between the basal lamina and the sarcolemma of the myofibre (Mauro, 1961). Satellite cells form a quiescent pool of cells that do not express genes or produce proteins (Kuang et al., 2008; McKinnell et al., 2008; Seale et al., 2000). Upon muscle injury satellite cells become activated during the remodelling phase. Activation of the satellite cells is caused by mechanical stretch to the myofibre itself, that induces the production of nitric oxide and consequently the release of HGF that in turn is able to activate

satellite cells via c-MET signalling (Tatsumi et al., 1998; Wozniak and Anderson, 2007; Wozniak et al., 2003). Another activator of satellite cells is Sphingosine-1-Phosphate (S1P) that is released from the inner leaflet of the plasma membrane by cleavage of sphingomyelin (Nagata et al., 2006). Activated satellite cells enter the cell cycle and proliferate as a transient amplifying pool of cells, the so-called myoblasts. After several rounds of proliferation, myoblasts exit the cell cycle and differentiate into committed precursor cells, the so-called myocytes, which finally fuse with each other to form myotubes. These different phases of adult myogenesis are summarized in Fig. 1.

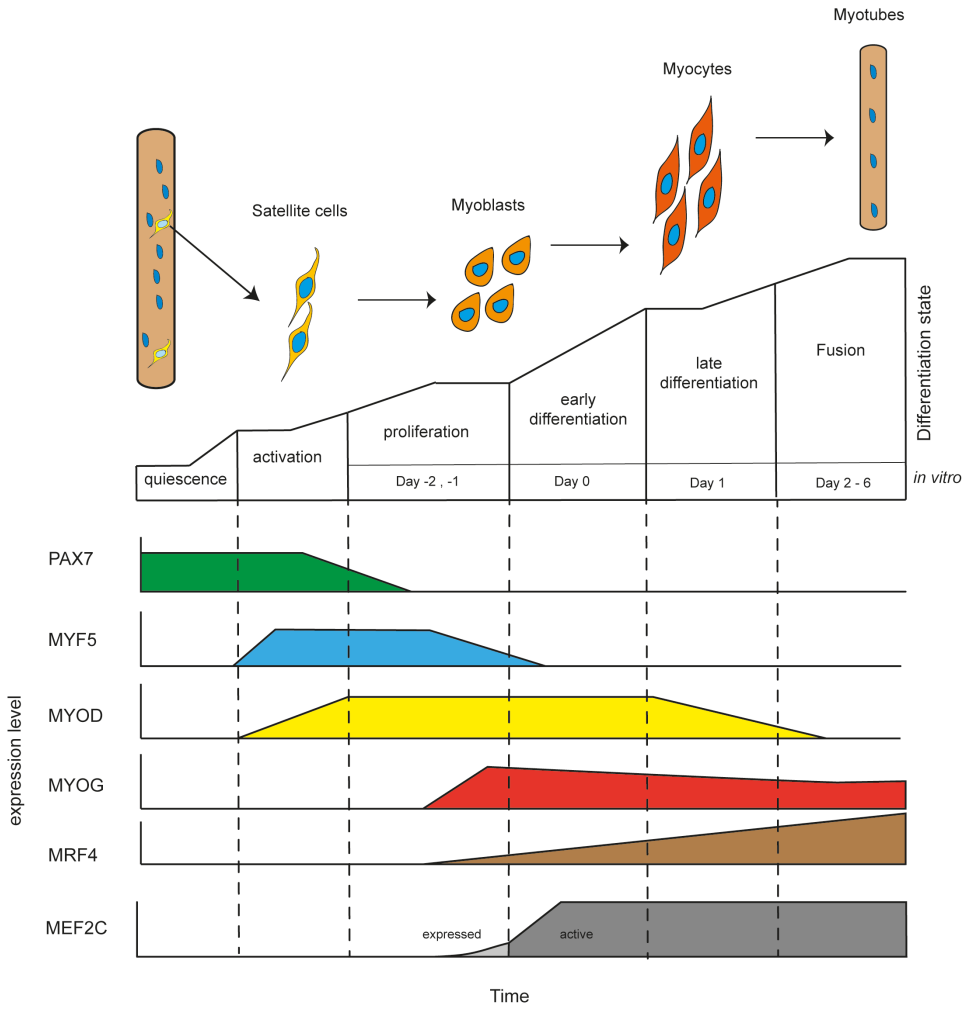

Figure 1 The different stages of adult myogenesis and the expression of myogenic transcription factors

Adult myogenesis is a multistep process that is regulated by a network of transcription factors. The expression of each transcription factor is regulated during the process. Adult myogenesis can be recapitulated *in vitro* from the proliferation phase until fusion. Figure modified from (Bentzinger et al., 2012).

1.2 Transcription control of adult myogenesis

Adult myogenesis is controlled by a network of transcription factors. To keep control over the network, the expression of each transcription factor is temporally regulated (Fig. 1). In adult myogenesis three families of transcription factors are involved. The paired box transcription factor PAX7, the myogenic regulatory factors (MRFs) MYF5, MYOD, MYF4 (myogenin), MRF4 and members of the MEF2 family of MADS transcription factors, in particular MEF2C.

1.2.1 The paired box transcription factor PAX7

The paired box transcription factor PAX7 is expressed in resting satellite cells and in proliferating myoblasts. Its expression is lost when myoblasts start to differentiate into myocytes. In Satellite cells, PAX7 fulfils several functions. On the one hand it is responsible for the survival of the cells and their self-renewal (Kuang et al., 2008; McKinnell et al., 2008; Seale et al., 2000). On the other hand it keeps the cells in an quiescent state by inducing the expression of ID2 and ID3 which repress the activity of MRFs (Kumar et al., 2009). It has been shown that mice lacking PAX7 do not have quiescent satellite cells and that isolated satellite cells from these animals cannot be cultivated (Seale et al., 2000). Moreover, forced expression of PAX7 in C2C12 myoblasts prevents the cells from differentiation (Zammit et al., 2006). However, although PAX7 is expressed in resting satellite cells and keeps them in a quiescent state, it also induces the expression of MRFs that drive differentiation (Bajard et al., 2006; Hu et al., 2008; Maroto et al., 1997). Thus its pro-differentiation action has to be restricted to a certain time point.

1.2.2 Muscle regulatory factors

Upon activation, satellite cells start to express muscle MRFs. The name muscle regulatory factor arises from their ability to drive non-muscle cells into myogenic differentiation when ectopically expressed (Braun et al., 1989; Edmondson and Olson, 1989; Weintraub et al., 1989). MRFs are part of a superfamily of basic-helix-loop-helix (bHLH) transcription factors and belong to class II, which show a tissue specific expression. In order to be active, MRFs need to either homo-dimerize or

hetero-dimerize with class I bHLH transcription factors. Class I bHLH transcription factors are ubiquitously expressed and include for example E-proteins such as E12/E47. All bHLH transcription factors bind the same consensus sequence called E-Box (Blackwell and Weintraub, 1990). Myogenic bHLH differ from other bHLH transcription factors by a “muscle recognition motif” in the basic domain (Brennan et al., 1991).

The first MRFs expressed after satellite cell activation are MYF5 and MYOD. Both proteins start being expressed at the same time, however they play distinct roles. Although MYF5 belongs to the myogenic factors, it is not able to promote differentiation on its own, thus it is proposed to function only in myoblasts where it promotes their proliferation (Kitzmann et al., 1998; Rawls et al., 1998; Ustanina et al., 2007; Valdez et al., 2000). Mice lacking MYF5 suffer from progressive myopathy and muscle regeneration defects that come along with increased myofibre diameter heterogeneity but also increased number of centralized nuclei and high levels of fibrosis. Interestingly the loss of MYF5 does not seem to have significant impact on satellite cell activation in contrast to the loss of MYOD that goes along with a strong increase in the number of satellite cells (Gayraud-Morel et al., 2007; Megeney et al., 1996).

Expression of MYOD starts upon activation of satellite cells. Its expression is maintained in cycling myoblasts and committed myocytes, but is lost in myotubes (Bentzinger et al., 2012). In myoblasts, MYOD has been shown to regulate the expression of ID3 that prevents the activity of other MRFs in order to promote myoblast proliferation (Wyzykowski et al., 2002). Paradoxically, MYOD is also required for the progression to terminal differentiation and induces the expression of other MRFs such as MYF4 and MRF4 (Gayraud-Morel et al., 2007; Ustanina et al., 2007).

Terminal differentiation is induced by the expression of myogenin (MYF4). Myogenin is expressed in myocytes and in myotubes (Bentzinger et al., 2012; Cheng et al., 1992). It induces cell cycle exit in myoblasts and the expression of structural proteins that are part of the contractile apparatus (Andrés and Walsh, 1996; Davie et al., 2007). Moreover, myogenin has been shown to be essential for the fusion process *in vivo* since myogenin null mice die perinatal due to a failure in

myotubes formation (Knapp et al., 2006; Nabeshima et al., 1993). In contrast to other MRFs which can at least partially compensate for each other, the loss of myogenin cannot be compensated by other MRFs (Rawls et al., 1995).

The last family member, MRF4, is expressed in myoblasts during early differentiation (Bentzinger et al., 2012). However, the exact role of MRF4 is still not known. A study in *Myf5/MyoD* double null mice has shown that MRF4 can rescue partially the phenotype resulting from the deletion of MYF5 and MYOD, showing that MYF5 and MYOD act upstream of MRF4 (Hasty et al., 1993).

1.2.3. The MADS box transcription factors

The transcription factor MEF2C belongs to the MADS domain transcription factors and is expressed at low levels in proliferating myoblasts. Its expression is strongly increased when early differentiation starts and is maintained in myocytes and myofibers (Gossett et al., 1989). Although it does not have any myogenic activity on its own, MEF2C acts as a transcription enhancer for other bHLH transcription factors such as MYOD (Molkentin et al., 1995). It promotes differentiation by inducing the expression of MRF4 and myogenin (Black et al., 1995; Cheng et al., 1992; Edmondson et al., 1992; Nabeshima et al., 1993) which in turn are responsible for final differentiation and fusion. This process is further enhanced by the fact that MEF2C also drives its own expression in a positive feedback loop to amplify MEF2C signalling (Wang et al., 2001). In addition, MEF2C drives cell cycle exit (Badodi et al., 2015; Chen et al., 2000), is involved in the formation of podosomes and the generation of the fusion pore prior to fusion (Sens et al., 2010) and drives myocyte fusion in an M-Cadherin-dependent manner (Duan et al., 2012). Finally MEF2C regulates the expression of structural proteins within the myotubes such as Myomesin I and II and Desmin (Potthoff et al., 2007). However, although MEF2C is needed for myocyte late differentiation and fusion it is already expressed in myoblasts (Liu et al., 2014; Mokalled et al., 2012).

1.3 Regulation of transcription factor activity during adult myogenesis

One key element in regulating adult myogenesis is to keep the separation between proliferation and differentiation. However, the temporal regulation of the expression

of the transcription factors that drive these steps is not sufficient. For example, MEF2C drives the expression of late differentiation genes, but is already expressed in proliferating myoblasts (Fig. 1). Thus, the activity of transcription factors that induce proliferation and those that promote differentiation must also be temporally regulated. For example, several mechanisms are known to regulate MEF2C activity during the course of myogenesis. One such mechanism is the control of its subcellular localization. MEF2C is exported from the nucleus when myoblasts are treated with anti- myogenic factor transforming-growth factor β (TGF-β), this possibly prevents MEF2C from interacting with MYOD and thus inhibits myogenesis (De Angelis et al., 1998). During proliferation MEF2C activity is also controlled by the retinoblastoma protein (Rb), which is phosphorylated during proliferation and it induces growth arrest in G1 by inactivating E2F when de-phosphorylated. MEF2C function depends on the binding to MYOD as well as to Rb (Novitch et al., 1999). In addition, during proliferation MEF2C is constantly degraded by the anaphase promoting complex/cyclosome (APC/C) after it is phosphorylated (Badodi et al., 2015). Furthermore, MEF2C transcriptional activity is also regulated by transcriptional co-activators such as steroid receptor co-activator GRIP-1 and p300. GRIP-1 has been shown to bind MEF2C and to potentiate its activity and to further recruit p300 to enhance this effect (Chen et al., 2000; Sartorelli et al., 1997). However, during proliferation, GRIP-1 co-activation of MEF2C is prevented by CDK4 by a yet unknown mechanism (Lazaro et al., 2002). Also transcriptional co-repressors such as histone deacetylases (HDACs), in particular class IIa HDACs play a role in MEF2C regulation. During proliferation, myoblasts express high levels of HDAC4 and HDAC5 which repress MEF2C activity. During differentiation, calcium-calmodulin signalling that is induced by the release of intracellular Ca^{2+} promotes HDAC4 and HDAC5 phosphorylation, which results in their export from the nucleus, thus de-repressing MEF2C activity (Lu et al., 2000a; McKinsey et al., 2000a).

Thus, several mechanisms ensure that MEF2C is inactive during the proliferation phase and active when cells differentiate. However, the regulation of MEF2C activity at the critical point where proliferation stops and differentiation starts is not fully understood. Interestingly in a yeast two-hybrid screen MEF2C was found to interact with the focal adhesion LIM domain protein TRIP6 (Orchard et al., 2014).

1.4 LIM domain proteins

The LIM domain is a cysteine rich motif that was named after the transcription factors Lin-11 Isl-1 and Mec3 in which it was first identified (Freyd et al., 1990). Spectroscopic analysis showed that LIM domains contain two specific zinc binding structures, which coordinates two zinc ions to form two so-called “zinc fingers” (Fig.2). In contrast to the zinc fingers found in transcription factors, LIM domains serve as protein-protein interaction domains and are not able to bind DNA (Kadrmas and Beckerle, 2004).

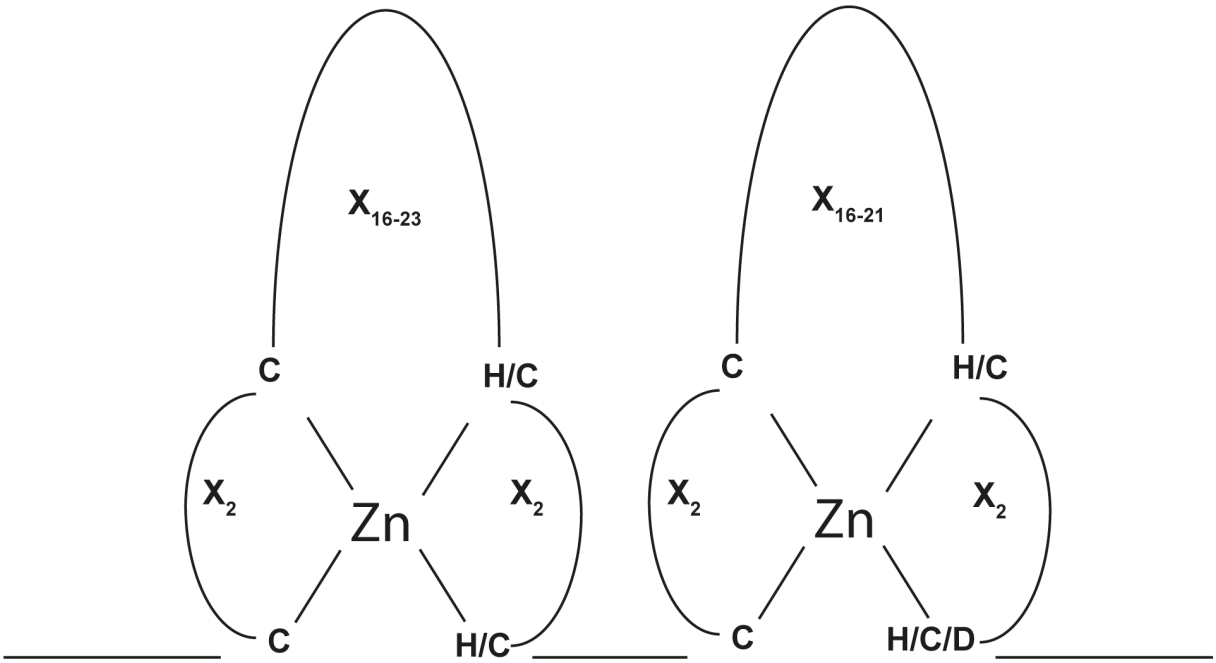

Figure 2 Consensus sequence and structure of LIM domains

LIM domain proteins can be divided into three different groups based on their sequence similarity and subcellular localization (Dawid et al., 1995). For an overview on the different groups see Kadrmas and Beckerle (2004). One of these groups contains primarily cytoplasmic proteins, most of which being associated with the cytoskeleton. Within this group, the so-called focal adhesion LIM domain proteins belong to two families, the Paxilin and Zyxin families. The Zyxin family is composed of seven members, Zyxin, Ajuba, LIMD1, LPP, WTIP, Migfilin and

TRIP6. As LIM domain proteins, they contain three tandemly arranged C-termimal LIM domains, as well as a N-terminal unstructured proline rich region and a highly conserved leucine rich sequence that harbours a functional nuclear export signal (NES). Therefore, members of the Zyxin family have a predominantly cytosolic localization. These proteins are enriched at sites of cell-matrix interaction, in particular focal adhesion, as well as at sites of cell-cell adhesion, and are known to regulate actin assembly and organisation, adhesion and migration (Smith et al., 2014). Surprisingly, focal adhesion LIM domain proteins also regulate the transcriptional activity of various transcription factors (Guo et al., 2006; Langer et al., 2008; Xu et al., 2003). Indeed, these proteins also harbour less characterized nuclear targeting regions and are therefore able to shuttle between the cytoplasm and the nucleus, where many act as transcriptional co-regulators (Nix and Beckerle, 1997).

1.5 The LIM domain protein TRIP6

TRIP6 belongs to the Zyxin family of focal adhesion LIM domain proteins. It was first identified in a yeast two-hybrid screen as Thyroid hormone receptor binding protein 6 in human cells (Lee et al., 1995). TRIP6 comprises 480 amino acids with a mass of 50.9 kDa. Like in the other members of the Zyxin family, three LIM domains are present in the C-terminus of TRIP6 (Yi and Beckerle, 1998). In the N-terminus, TRIP6 harbours a functional NES, and is therefore located in the cytoplasm and enriched at sites of focal adhesion (Wang and Gilmore, 2001). The exact function of TRIP6 is still not completely revealed but it is known to play a role in cell adhesion and migration (Bai et al., 2007; Lai et al., 2005, 2007). Although TRIP6 contains a functional NES, it also contains poorly defined nuclear targeting sequences, together with sequences that can activate transcription (Wang and Gilmore, 2001) . Thus TRIP6 was proposed to shuttle from the cytoplasm to the nucleus to regulate transcription (Wang and Gilmore, 2001). However, although some reports propose TRIP6 to act as transcriptional regulator (Lin and Lin, 2011; Solaz-Fuster et al., 2006), there has been no evidence that TRIP6 really shuttles between the cytoplasm and the nucleus.

1.6 nTRIP6, the nuclear isoform, of the LIM domain protein TRIP6

Our group has identified a smaller isoform of TRIP6, only present in the nucleus, and therefore called nTrip6 (Kassel et al., 2004). TRIP6 showed no evidence of nuclear shuttling and its nuclear functions were attributed to nTRIP6 (Diefenbacher et al., 2008, 2010, 2014; Kassel et al., 2004). nTRIP6 is generated through the usage of an alternative translational initiation codon within the NES encoding sequence (Fig. 3). The usage of this second AUG leads to a truncated and non-functional NES. Thus nTRIP6 is located within the nucleus (Winter 2007). In the nucleus nTRIP6 acts as transcriptional co-activator for several transcription factors such as AP-1, NF-κB and the glucocorticoid receptor (GR) (Diefenbacher et al., 2008, 2010, 2014; Kassel et al., 2004). nTRIP6 interacts with these transcription factors via its LIM domains, is recruited together with the transcription factors to the regulatory regions of target genes and increases their transcription by mediating the promoter recruitment of other co-activators such as THRAP3 (Diefenbacher et al., 2008, 2010, 2014; Kassel et al., 2004).

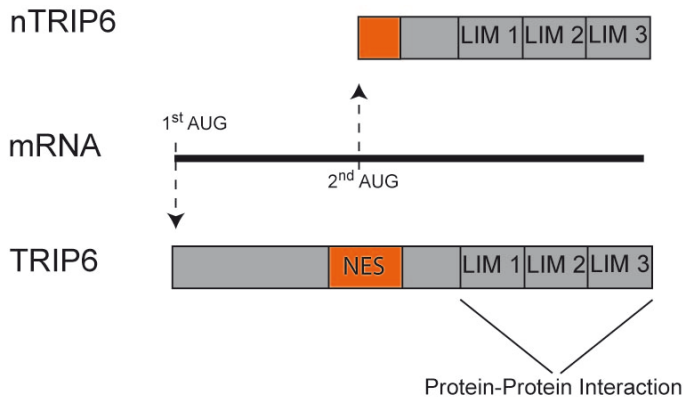

Figure 3 TRIP6 and its nuclear isoform nTRIP6

TRIP6 and nTRIP6 are translated from two different initiation codons in the same mRNA. Translation start at the first start (AUG) is leading to TRIP6, the long isoform containing a functional NES. Therefore, TRIP6 is localized in the cytosol. The shorter isoform nTRIP6 is translated from the second AUG located within the NES encoding sequence. It does not contain a functional NES anymore and is therefore localized in the nucleus.

In a Yeast-two hybrid screen the transcription factor MEF2C was reported to interact with TRIP6. However, since the smaller isoform of TRIP6 called nTRIP6 is a known co-regulator for transcription factors it is possible that nTRIP6 rather than TRIP6 acts as co-regulator for MEF2C.

1.7 Aim

The precise regulation of the myogenic transcription factor MEF2C transcriptional activity at the critical transition between myoblast proliferation and differentiation is not fully understood. In a yeast two-hybrid screen, TRIP6 has been reported to interact with MEF2C (Orchard et al., 2014), suggesting that it may regulate MEF2C activity. However, since the nuclear isoform nTRIP6 is a known co-regulator for other transcription factors, it is possible that not TRIP6 but rather nTRIP6 acts as co-regulator for MEF2C.

Thus, the aim of this study was to investigate whether nTRIP6 acts as transcriptional co-regulator for MEF2C, and if so by which mechanism. Moreover, given the central role played by MEF2C during adult myogenesis, I studied a possible role for nTRIP6 in regulating adult myogenesis *in vitro* and skeletal muscle regeneration *in vivo*.

2 Material and Methods

2.1 Material

2.1.1Consumables

All cell culture dishes and plates if not stated otherwise were obtained from Greiner Bio-One, Frickenhausen

2.1.2 Chemicals

Chemicals	Company
Collagen I (rat tail)	Corning
Collagenase	Sigma
Notexin	Latoxan
Tamoxifen 99%	Sigma

All other chemicals if not stated otherwise were purchased from Carl Roth, Karlsruhe.

2.1.3 Kits

Kits	Company
Amaxa Nucleofection V	Lonza
Amersham ECL Prime	GE Healthcare
QUIAGEN Maxi Plasmid Kit	Quiagen
GeneJet Gel Extraction	Thermo Fischer

2.1.4 Hardware

Device	Company
LSM510Meta	Zeiss
Chemidoc X Touch Imaging Sytem	Biorad
Trans Blot SD Cell	Biorad

2.1.5 Enzymes

All enzymes if not stated otherwise were purchased from NEB or Promega.

2.1.6 Antibodies

Antibody	Isotype	Concentration	Company	Ordernumber
α-actinin	Mouse	1:1000(WB)	Santa Cruz	sc-59953
α-mCherry	Rabbit	1:1000 (IF)	Abcam	ab167453
α-mCherry	Mouse	1:1000 (WB)	Abcam	ab125096
α-GR	Rabbit	1:1000 (WB)	Santa Cruz	sc-1004
α-HA	Rat	1:100(IF) 1:1000 (WB)	Sigma	11867423001
α-HDAC5	Goat	1:100(IF) 1:1000 (WB)	Santa Cruz	sc-5252
α-Laminin	Rabbit	1:1000(IF)	Sigma	L9393
α-Laminin 2 alpha	Rat	1:1000(IF)	Abcam	ab11576
α-Mef2C	Rabbit	1:100(IF) 1:1000 (WB)	Cell Signalling	D80C1
α-MyoD	Rabbit	1:100(IF)	ThermoFischer	PA5-23078
α-myogenin	Mouse	1:100(IF) 1:1000 (WB)	DSHB	F5D-s
α-myHC (MYH3)	Mouse	1:100(IF) 1:1000 (WB)	DSHB	F1.652
α-p21	Mouse	1:100(IF) 1:1000 (WB)	BD Bioscience	556430
α-Trip6	Rabbit	1:100(IF) 1:1000 (WB)	Self made	
Secondary antibodies				
α-mouse Alexa488	Goat	1:10000	ThermoFischer	A-11001
α-mouse Alexa546	Goat	1:10000	ThermoFischer	A-11030

α-rabbit Alexa 488	Goat	1:10000	ThermoFischer	A-11059
α-rabbit Alexa 546	Goat	1:10000	ThermoFischer	A-10040
α-rat Alexa 546	Goat	1:10000	ThermoFischer	A-11081
α-mouse- HRP		1:2000	Dako	P0260
α-rabbit-HRP		1:2000	Dako	P0448
α-rat-HRP		1:2000	Dako	P0450

2.1.7 Buffers

General buffers

TAE	40 mM Tris acetate, 1 mM EDTA[pH 8.2 - 8.4 (at 25°C)]
RIPA:	50mM Tris pH 7.5; 150mM NaCl; 1% NP-40; 0.5% NaDoc; 0.5% SDS
Gly Gly	25mM Glycylglycin; 15mM $MgSO_4$; 4mM EGTA; pH 7,8
NID	10mMTrisHCl pH 8.3; 50mM KCl; 2,5mM $MgCl_2$; 0,1mg/ml Gelatine; 0.45%NP40;0,45%TWEEN20

ChIP buffers

Swelling Buffer	25mM Hepes pH7.8; 1.5mM $MgCl_2$; 10mM KCl; 0.1%NP-40; 1mM DTT; 0.5mM PMSF; 1xPIC
Sonication Buffer	50mM Hepes pH 7.9; 140mM NaCl;1mM EDTA 1% TritonX-100;0,1%SDS; 0.5mMPMSF; 1x PIC
Dilution buffer	0,01%SDS, 1% Triton-X-100, 2mM EDTA, 16.7mM TrisHCl pH8.1, 167mM NaCl
Low salt buffer	0,1%SDS, 1%Triton-X-100, 2mMEDTA, 20mM TrisHCl pH8.1, 150mM NaCl

High salt buffer	0.1%SDS, 1% Triton X-100, 2mM EDTA, 20mM TrisHCl pH8.1, 500mM NaCl
Elution Buffer	Normal: 0.1M $NaHCO_3$, 1% SDS (add fresh) Re-ChIP: 10mM DTT

Immunofluorescence staining

Fixation buffer	2% Paraformaldehyde in PBS -/- pH 7.2
Permeabilization buffer	0.5% Triton X-100 in PBS -/-
Blocking buffer	5% BSA in PBS -/- pH 7.2

Protein measurement according to Lowry

Lowry 1	20g/L Na2CO3; 4g/L NaOH in ddH2O
Lowry 2	1% CuSO4
Lowry 3	2% NaK Tartrat
Lowry 4	1% Lowry II buffer; 1% Lowry III buffer in Lowry I buffer

SDS-PAGE and Western Blot

Separating gel	10% Acrylamid:Bisacrylamid; 375mM TrisHCL (pH 8.8); 0.1% SDS; 0.1% APS; 0.04% TEMED
Stacking gel	5% Acrylamid:Bisacrylamid; 125mM TrisHCL (pH 6.8); 0.1% SDS; 0.1% APS; 0.1% TEMED
Electrophoresis buffer	25mM Tris; 192mM Glycin; 0.1% SDS
2x Sample buffer	125mM TrisHCL (pH 6.8); 4% SDS; 20% Glycerol; 0.01% Bromphenol blue; 2% 2-Mercaptoethanol
TBST	20mM Tris; 150mM NaCl; pH 7,6)+ 0,05% TWEEN 20
Blotting buffer	20mM Tris; 192mM Glycine; 10% Methanol
Blocking buffer	5% skimmed milk powder; TBS

Luciferase assay and liquid β-Galactosidase assay

Reaction Buffer	2mM ATP; 1mM DTT in GlyGly Buffer
Substrate	1mM Luciferin 1:10 in GlyGly Buffer
Solution A	Sodium Phosphate 0,1M
Solution B	ONPG 1mg/ml
Solution C	100mM $MgCl_2$, 4,48M β-Mercaptoethanol

2.1.8 Bacterial strains and growth media

2.1.8.1 Bacterial strains

E. coli DH5α (Genotype: supE44ΔlacU169 (ΦlacZΔM15) hsdR7 recA1 gyrA96 thi-1 relA1)

2.1.8.2 Bacterial growth media

Luria Both (LB):	Yeast extract	10g/l
	NaCl	5g/l
	Tryptone	5g/l
	pH 7.5	

2.1.9 Cell lines and cell culture media

2.1.9.1 Cell line

C2C12	Mus musculus muscle myoblast cells (ECACC No.: 91031101)

2.1.9.2 Cell culture media

C2C12 growth medium	DMEM + 10% FCS
C2C12 differentiation medium	DMEM + 0.5% Horse Serum

2.1.10 Plasmids

pcDNA3.1(+)	Empty vector, basic mammalian expression cloning vector
pcDNA3.1(+)HA- mCherry-NLS	Contains the cDNA of a functional Nuclear Localization Signal (NLS) sequence fused to the cherry-red fluorescent protein (mcherry) under the control of a CMV promoter. (provided by Margarethe Litfin, ITG, KIT)
pcDNA3.1(+)HA-mCherry-NES	Contains the cDNA of a functional Nuclear Export Signal (NES) sequence fused to the cherry-red fluorescent protein (mcherry) under the control of a CMV promoter.

	(provided by Markus Diefenbacher, ITG, KIT)
pcDNA3.1(+) –mCherry-NLS-ID 1	Contains the cDNA of nTrip6$^{175-187}$ fused to a NLS sequence and to the cherry-red fluorescent protein (mCherry) under the control of a CMV promoter. (provided by Markus Diefenbacher, ITG,KIT)
pcDNA3.1(+)-mCherry-NLS-ID1c	Contains the scrambled cDNA of nTrip6$^{175-187}$ fused to a NLS sequence and to the cherry-red fluorescent protein (mCherry) under the control of a CMV promoter. (provided by Markus Diefenbacher, ITG,KIT)
pcDNA3.1(+)-mCherry-NLS-ID2	Contains the cDNA of nTrip6$^{257-270}$ fused to a NLS sequence and to the cherry-red fluorescent protein (mCherry) under the control of a CMV promoter. (provided by Markus Diefenbacher, ITG,KIT)
pcDNA3.1(+)-mCherry-NLS-ID2c	Contains the scrambled cDNA of nTrip6$^{257-270}$ fused to a NLS sequence and to the cherry-red fluorescent protein (mCherry) under the control of a CMV promoter. (provided by Markus Diefenbacher, ITG,KIT)
pcDNA3.1(+)-MEF2C	Contains the cDNA of MEF2C

	(provided by Eric N Olson, University of Texas Dallas)
pGL3-TATA-DesMef3-Luc	Contains the Luciferase gene driven by three copies of the Mef2C response element (provided by Eric N Olson, University of Texas Dallas)
pcDNA3.1(+)β-Galactosidase	Contains the cDNA of the enzyme β-Galactosidase
pcDNA3.1(+)HAnTrip6	pcDNA3.1(+) vector containing the cDNA of mouse nTrip6 fused to an HA tag at its N-terminus. (provided by Oliver Dahley ITG,KIT)
pcDNA3.1(+)HAnTrip6ΔID1	pcDNA3.1(+) vector containing the cDNA of mouse nTrip6 fused to an HA tag at its N-terminus lacking the amino acids from 175-187.
pcDNA3.1(+)HAnTrip6ΔID2	pcDNA3.1(+) vector containing the cDNA of mouse nTrip6 fused to an HA tag at its N-terminus lacking the amino acids from 257-270.
pcDNA3.1(+)HA-MCS-VN	Contains the coding sequence 1-172 of the N-terminal part of Venus fluorescent protein (VFP) under the control of a CMV promoter.
pcDNA3.1(+)HA-MCS-VC	Contains the coding sequence 155-238 of the C-terminal part of Venus fluorescent protein (VFP) under the control of a CMV promoter.

The following constructs were cloned in the MCS of the above described vectors using KpnI and XbaI:

pcDNA3.1(+)HATrip6-VN

pcDNA3.1(+)HAnTrip6-VN

pcDNA3.1(+)HAnTrip6-VC

pcDNA3.1(+)HAnTrip6-Nter-VN

pcDNA3.1(+)HAnTrip6-LIMonly-VN

pcDNA3.1(+)HAnTrip6ΔID1-VN

pcDNA3.1(+)HAnTrip6ΔID2-VN

pcDNA3.1(+)HAMef2C-VC

pcDNA3.1(+)HAHDAC4-VN

pcDNA3.1(+)HAHDAC5-VN

2.1.11 Primers

Genotyping

Trip6 $^{flox/flox}$

mTrip6-28F	tcacctttctcccttgcctgcctg
mTrip6-29R	ggtacccccggaggctgataacag

Pax7 $^{CRE\ ERT2}$

gtPax7CreERT2for	GCTGCTGTTGATTACCTGCC
gtPax7CreERT2mrev	CAAAAGACGGCAATATGGTG
gtPax7CreERT2wtrev	CTGCACTGAGACAGGACCG

ChIP

pmDesMef2QChIPfor	CCCAGAACGCCTCTCCTGTACCTT
pmDesMef2QChIPrev	CAGCCGTCTCCCTAGCAGCAACA
pmTnni2QChIPfor1	GCTGGCATCTTGAACTCGTC
pmTnni2QChIPrev1	CCAGGCCACACAGAAGAAC
pmMyoglobinQChIPfor	GGGCTTGTGCAAGTCCAGACAGTG
pmMyoglobinQChIPrev	CCCTTCCTGCTACCGTGCTCAAC
pmMyom2QChIPfor	GAGCAGAGTACCCTGGGACG
pmMyom2QChIPrev	TTATGGCCAGAGGAGGTGCTA

2.1.12 siRNA

Trip6 siRNA	5’-cag ucu gga ugc uga gau aga (dTdT)-3’
Control siRNA	5’-agg uag ugu aau cgc cuu gtt (dTdT)-3’

2.1.13 Fluorescent Dyes

DRAQ5^{TM}

Synthetic fluorescent dye with a high affinity to DNA, thus it can be used to highlight the nuclei of living and fixed cells or tissue.

Excitation (Max): 647nm

Emission (Max): 670nm

2.2 Methods

2.2.1 Animals and animal handling

Experiments were performed on C57/BL6 mice. Use and care of the animals was approved by German authorities (Tierschutzkommission of the Regierungspräsidium Karlsruhe, licenses G-232/11 and G261/15) according to national law (TierSchG §7).

2.2.1.1 *Trip6* conditional knockout mouse (Cre/LoxP System)

The *Trip6* conditional knockout mouse was generated by crossing two different mouse lines: a *Trip6* floxed line, in which loxP sites were introduced after exons 1 and 9 of the *Trip6* gene (Markus Winter, ITG KIT), and a mouse expressing the tamoxifen-inducible Cre-Recombinase (Cre-ERt2) under the control of the pax7 promoter, thus exclusively expressed in satellite cells (Murphy et al., 2011). Cre-Recombinase is fused to the ligand binding domain of the Estrogen Receptor (ER) carrying the T2 mutation, which renders the receptor insensitive to endogenous Estrogen but sensitive to Tamoxifen. Upon Tamoxifen binding to Cre-ERT2, the fusion protein shuttles to the nucleus where the Cre-Recombinase recognizes the loxP sites and deletes the *Trip6* gene in satellite cells (Fig. 4)

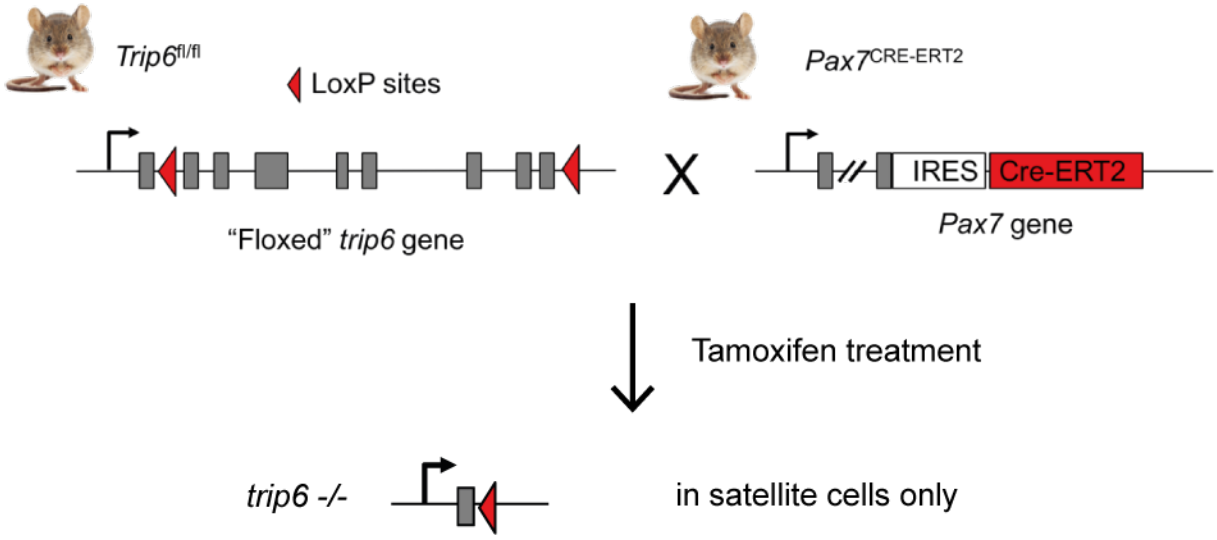

Figure 4 Satellite cell-selective conditional trip6 knockout mouse

2.2.1.2 Genotyping

Mouse tails were digested overnight in 200µl NID-buffer + 2µl (10mg/ml) Proteinase K at 55°C. Next day Proteinase K reaction was stopped by heating up to 95°C for 30min. Samples were then centrifuged for 15min at 13000rpm at 4°C.

Genotyping was performed via PCR using the primers listed in 2.1.10.

Trip6 $^{flox/flox}$		**PCR program**	
5x Buffer	10µl	95°C	5min
10mM dNTP	1µl	95°C	1min
10µM Primer	2µl	71°C	1min
Taq Polymerase	0.25µl	72°C	1 min
DNA	4µl	35-40 cycles	
Total Volume	50µl	72°C	10min
		8°C	store

Pax7-Cre-ERT2		**PCR program**	
5x Buffer	10µl	95°C	5min
10mM dNTP	1µl	95°C	1min
10µM Primer	2µl	62°C	1min
Taq Polymerase	0.25µl	72°C	1 min
DNA	4µl	35-40 cycles	
Total Volume	50µl	72°C	10min
		8°C	store

2.2.1.3 Treatment with Tamoxifen

Mice were injected intraperitoneally on 5 consecutive days with 5µl/g body weight Tamoxifen from a 10mg/ml stock solubilized in peanut oil.

2.2.1.4 Degeneration of murine *M soleus*

Degeneration of soleus muscle was induced as described in (Danieli-Betto et al., 2005, 2010) Animals were anesthetized by an intraperitoneal injection of Ketamin and Xylasin (100mg/kg body weight Ketamine, 16 mg/kg body weight Xylazine) inject 0.1 ml/10 g body weight). 10µl of a 5ng/µl Notexin solution diluted in sterile PBS-/- were injected unilaterally in the exposed soleus muscle, through a small

cutaneous incision. Animals were sacrificed by cervical dislocation after 7 days, 10, 14 and 28 days of regeneration.

2.2.1.5 Preparing and sectioning skeletal muscle tissue

Mice were sacrificed by cervical dislocation at 3-6 weeks of age. *M. soleus* was dissected by cutting the tendons, then fixed in a stretched position and frozen in liquid nitrogen. The muscle was cut in two halves with a scalpel. Half of the muscle was then fixed on a mounting carrier using O.C.T. compound (Tissue-Tek) and 10µm thick cryosections were cut using a cryostat. These sections were then transferred onto a glass slide for further processing.

2.2.1.6 Immunofluorescence staining of muscle tissue sections

Muscle sections were fixed for 5 minutes in fixation buffer at room temperature. After fixation the sections were washed 3 times for 5 minutes in PBS-/-. For permeabilization the sections were incubated for 10 minutes in 0.5%Triton-X-100 in PBS-/- and afterwards washed again. Sections were blocked in blocking solution for 1h at room temperature. The 1st antibody was diluted at an appropriate concentration in blocking solution and incubated on the section overnight at 4°C. On the next day sections were washed and the appropriate secondary antibody was applied for 1h at room temperature in the dark. After washing three times with PBS -/- the sections were mounted with Mowiol 4-88 and the sections were subjected to analysis.

2.2.1.7 Microscopy and Quantification of the cross section area of regenerating myofibers

Muscle sections were stained according to 2.2.5 with an antibody against laminin to visualize the basal lamina of the fibres. Microscopy images were acquired using a 10X Plan- Apochromat objective. On each picture single myofibres were segmented automatically and the minimum Feret's Diameter was measured using the ImageJ Software.

2.2.1.8 Preparation of isolated fibres of Extensor digitorum longus (EDL)

Mice were sacrificed by cervical dislocation. EDL was dissected as described in 2.2.1.5. The digestion was performed in DMEM + 0.2% Collagenase for 1h at 37°C. Afterwards the fibres were dissociated by pipetting the muscle up and down in a

cut Pasteur pipet with decreasing opening. The released fibres were collected and further processed as described in 2.2.1.6.

2.2.2 Cell culture methods

2.2.2.1 Cell culture conditions

All cells were cultured at 6% CO_2, 95% humidity at 37°C. Manipulation of cells was performed under a sterile hood. Media, buffers and glassware were sterilised before work (120°C, 1.4 bar, 20min).

2.2.2.2 Passaging and seeding of cells

After removing the medium the cells were washed with PBS lacking Ca^{2+} and Mg^{2+}. Then the cells were treated with a Trypsin solution and incubated for 2 minutes in the incubator. The trypsin reaction was stopped by adding medium to the dish and the cells were resuspended carefully. Thereafter the cells were counted and transferred to a new dish. To determine the cell number a Neubauer chamber was used.

Format	**Cell number**
15 cm Dish	$2,5x10^5$
24 well plate	$1x10^4$
IBIDI	$5x10^3$

2.2.2.3 Transfection of C2C12 cells with Promofectin

Cells were seeded according to 2.2.2.2 and transfected according to manufacturer's protocol, with appropriate amounts of DNA

Format	**Amount DNA [ng]**	**Volume Medium [µl]**	**Transfection Mix [µl]**
24 Well plate	1000	500	50
IBIDI	400	150	25

2.2.2.4 Transfection of C2C12 cells with Amaxa

$1x10^6$ cells were transfected with either 6µg of plasmid DNA or 600pmol of siRNA according to manufacturer's protocol. Before seeding cells were stained with trypan blue and counted to estimate the number of viable cells.

2.2.3 Protein methods

2.2.3.1 Protein isolation (in RIPA buffer)

Cells were washed twice with ice cold PBS, scraped and transferred into 15ml Falcon tube. The cells were centrifuged at 1,200rpm, 4°C for 3min and the supernatant was discarded. The cell pellet was resuspended in 20µl RIPA buffer (supplemented with PIC) and sonicated for 5min (low intensity)

2.2.3.2 Measurement of total protein concentration according to Lowry

250µl Lowry I-buffer were added to 4µl of the sonicated protein lysate. 500µl Lowry IV-buffer was added to all samples and incubated 5min at RT. 50µl Folin was added and incubated for 30 minutes at RT. The protein concentration was assessed by spectrometry at 595nm wavelength and the extinction was measured and compared to a standard curve.

2.2.3.3 Separation of Proteins via SDS-Page

Proteins were separated according to their size using an electrical field (Laemmli, 1970)The polyacrylamid gels were casted according to (Sambrook et al., 1989). The gels were run in a mini gel system (Hoefer, San Francisco, USA) at 100V for 1h.

2.2.3.4 Western Blotting

After the separation on the gel, the proteins were transferred to an Immobilon-P membrane soaked in methanol and activated before used. For the transfer a semi-dry blotter at 1mA/cm^2 for 1h was used with western blot buffer. After the blotting the membrane was incubated in blocking buffer for 1h at room temperature. Then the primary antibody diluted in the blocking buffer was incubated on the membrane over night at 4°C under constant shaking. The next day the membrane was washed with TBST three times for 5 minutes at room temperature. The secondary antibody (conjugated with HRP) was diluted in blocking buffer and the membrane was incubated with the antibody for one hour at room temperature. The membrane was then washed with TBST three times for 5 minutes. The detection of the specific signal of the HRP was done using an ECL Western Blot kit and the Biorad ECL Imaging machine

2.2.4 DNA methods

2.2.4.1 PCR (Pfu-DNA-Polymerase)

A sequence specific primer pair was used in order to amplify DNA-fragments by using the Pfu-DNA-polymerase (Promega). 0.1µg of DNA, 0.4mM of each Primer, 10% DMSO, 0.2mM dNTPs, 1U Pfu-DNA-Polymerase and the appropriate volume of 10x PCR-Buffer (Promega) were mixed on ice. The reaction was carried out in a Perkin-Elmer-Thermocycler (Norwalk, USA). Annealing temperature and time was adapted to each PCR. Elongation was carried out at 72°C and the time adapted to the size of the fragment (2min/kb).

2.2.4.2 DNA digestion

2 units of the corresponding restriction endonuclease were used to digest 1µg of plasmid DNA. The total volume of the reaction mix was 10 times the volume of the enzyme/glycerol solution. In order to achieve the optimal salt and pH-conditions for the enzymes to work best, a corresponding buffer was used, that was 10 times concentrated. The reaction mix was incubated, if not stated otherwise by the supplier, at 37°C for 2h.

2.2.4.3 Electrophoresis

In order to separate DNA fragments according to their size, DNA was subjected to an electric field within a 1% agarose gel matrix. To produce a 1% agarose gel the corresponding amount of agarose was weighed and dissolved in 1x TAE buffer. After dissolving the agarose and cooling down Ethidium bromide was added and the gel was cast. The separation was achieved by applying an electric current with a voltage of 100V. The separated DNA was visualized using UV-light.

2.2.4.4 Isolation of DNA Fragments out of Agarose Gels

Fragments of the size of interest were cut out of the gel using a scalpel at an UV-light table. The DNA was purified out of piece of gel using the GeneJet Gel Extraction Kit.

2.2.4.5 Phenol-Chloroform extraction

DNA solution was mixed with 1vol of Phenol:Chlorofom:Isoamylalcohol (24:1:1) and vortexed for one minute. Centrifugation was performed with 14000rpm for 5 minutes. DNA containing phase was transferred into a fresh reaction tube and

mixed with 1vol of Chloroform: Isoamylalcohol (24:1) and vortexed. After that centrifugation was performed with 14000rpm for 5 minutes and the aqueous phase was transferred to a fresh reaction tube and mixed with 2.5vol 100%EtOH with 20µg of Glycogen and 1/15 Na-actetate. Precipitation was performed for at least one hour at -20°C. Centrifugation was performed with 14000rpm at 4°C for 30 minutes DNA pellet was washed with 75% EtOH and after drying resuspended in 20-50µl ddH_2O.

2.2.4.6 Ligation of DNA-fragments

The ligation reactions were performed using the T4 DNA ligase according to manufacturer's instruction (NEB). The reaction mix was incubated O/N at 16°C.

2.2.4.7 Colony PCR

A colony PCR was performed in order to screen bacterial clones. Neither the PCR program nor the PCR mix was altered. But instead of adding DNA, an *E. coli* clone was picked with a yellow pipette tip and first transferred into the PCR mix and then onto a fresh agar plate containing the appropriate selection marker

2.2.4.8 Transformation of *E. coli* (DH5α)

100µl of chemically competent *E. coli* (DH5α) were thawed on ice and 0.1µg of plasmid DNA was added. The mix was incubated on ice for 30min and then the bacteria were subjected to heat shock treatment for 30sec at 42°C in a heating block. The bacteria were then placed on ice for 2min and 1ml LB-medium was added. The transformed bacteria were incubated at 37°C for 1h before adding the selection marker. For selection, the bacteria were plated onto LB agar plates that contained the selection marker or were directly transferred into 100ml of LB-medium containing the selection marker. The bacteria were allowed to grow for 24h at 37°C.

2.2.5 Assays

2.2.5.1 Bimolecular fluorescent complementation assay (BiFC)

The BiFC (Hu et al., 2002) assay is a live cell protein-protein interaction assay based on the complementation of the fluorescence of a fluorescent protein (Venus or YFP) split into its C-terminal and N-terminal halves that are each fused to the two proteins of interest. If the two proteins interact with each other, the two halves

of the fluorescent protein are in close proximity and can fold as the native protein, and thus fluorescence can be observed. C2C12 cells were transfected according to 2.2.2.3. The next day medium was changed and the cells were subjected to microscopic analysis using the LSM510 Meta and a 40x Apochrom oil Objective.

2.2.5.2 Luciferase Reporter Gene Assay

C2C12 cells were seeded according to 2.2.2.2 and grown overnight. The next day the cells were transfected using Promofectin as described in 2.2.2.3. Up to 400ng of plasmid DNA were transfected and 100 ng of the reporter construct pGL3-TATA-DesMef3 and 50ng of pcDNA3.1β-galactosidase as control. The cells were incubated for 48h in a cell incubator at 6% CO_2, 95% humidity at 37°C. After incubation the cells were washed twice with ice cold PBS (-/-) and lysed with 100µl Passive Lysis buffer (Promega) for 15min on ice. After lysis, 20 µl of the lysed cells were transferred into a white 96well plate and subjected to luciferase measurement. 70µl of the reaction buffer together with 20µl substrate were injected automatically in each well. The luminescence was measured for 2 seconds.

2.2.5.3 Liquid β-Galactosidase Assay

20µl of cell lysate from 2.2.5.2 were mixed with the reaction solution (See buffers) Incubation was performed for 10-30min at 37°C in the dark. Quantification was performed in a photometer measuring at 420nm.

2.2.5.4 Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed on C2C12 cells seeded at a density of $5x10^5$ in a 15cm dish and grown for 48h. Cells were crosslinked for 10 min with formaldehyde (540µl in 20ml DMEM). After washing with cold PBS -/- the crossliniking was stopped by adding 10ml of 1x Glycin Buffer (Active Motif ChIP-IT Express) for 5 minutes. After washing with ice cold PBS-/- cells were harvested in 20ml ice cold PBS supplemented with 0.5mM PMSF and centrifuged at 1200rpm for 5 minutes at 4°C. The cell pellet was resuspended in 10 vol Swelling buffer and kept on ice for at least one hour. Centrifugation was performed at 2000rpm for 5 min and the cell pellet was resuspended in 4 times 350µl Sonication buffer. The chromatin was sheared into 200-1000 bp fragments by sonication with 30sec pulses separated by 30sec pauses for 1h at middle intensity (Bioruptor, Diagenode, Liège, Belgium). To determine shearing efficiency, 5µl of the sheared chromatin

were diluted with 15µl ddH_2O. The sheared chromatin was subjected to gel electrophoresis on a 2% agarose gel. To estimate the amount of chromatin the cell lysate was measured using the Nano Drop. For immunoprecipitation 65µg chromatin was mixed with 1350µl ChIP dilution buffer and 2.5µg of the antibody and incubated O/N at 4°C on a rotor. The next day 20µl of protein G-conjugated magnetic beads were incubated with the immunoprecipitated chromatin for 2h at 4°C on a rotor. The beads were then washed once with low salt buffer, 4 times with high salt buffer and then once with 1x TE buffer (1000µl). All washing steps were performed for 5 minutes under rotation at 4°C. After the last washing step the beads were incubated with 400µl elution buffer for 10 minutes at 65°C. The supernatant was transferred into a fresh Eppendorf tube, 21µl NaCl (4M) were added and the mix was incubated O/N at 65°C under shaking. The next day, DNA was purified using Phenol-Chloroform extraction (2.2.4.5) and the precipitated DNA was dissolved in 50µl ddH_2O.

2.2.5.5 Double Chromatin immunoprecipitation or ReChIP

After the first ChIP, the ReChIP was performed by eluting the chromatin with 100µl DTT (10mM) for 30 minutes at 37°C and shaking. Afterwards the eluted chromatin was diluted in ChIP dilution buffer, and the second immunoprecipitation was performed as described in 2.2.5.4.

2.2.6 Statistical analysis

Where indicated, significant differences were assessed by t-test analysis, with values of $P<0.05$ sufficient to reject the null hypothesis.

For the animal experiments, multiple comparisons between groups were made using ANOVA and Tukey's post-hoc analysis.

3 Results

3.1 nTRIP6 acts as a co-repressor for MEF2C

A search for MEF2C interacting proteins in the IntAct Database (Orchard et al., 2014) revealed TRIP6 as an interaction partner for MEF2C. If indeed TRIP6 co-regulates MEF2C it must be present in the nucleus. However, proteins from this family of LIM domain proteins all have an NES and are therefore mostly cytosolic. Nevertheless, some of them have been detected at low levels in the nucleus or can shuttle between the cytoplasm and the nucleus (Kadrmas and Beckerle, 2004; Nix and Beckerle, 1997). Therefore, I tested whether TRIP6 is present in the nucleus of C2C12 myoblasts as a prerequisite to act as a co-regulator for MEF2C. To do so I performed immunofluorescence staining using an antibody against TRIP6. The staining shows a strong cytosolic staining and also a weaker nuclear staining (Fig. 5a).

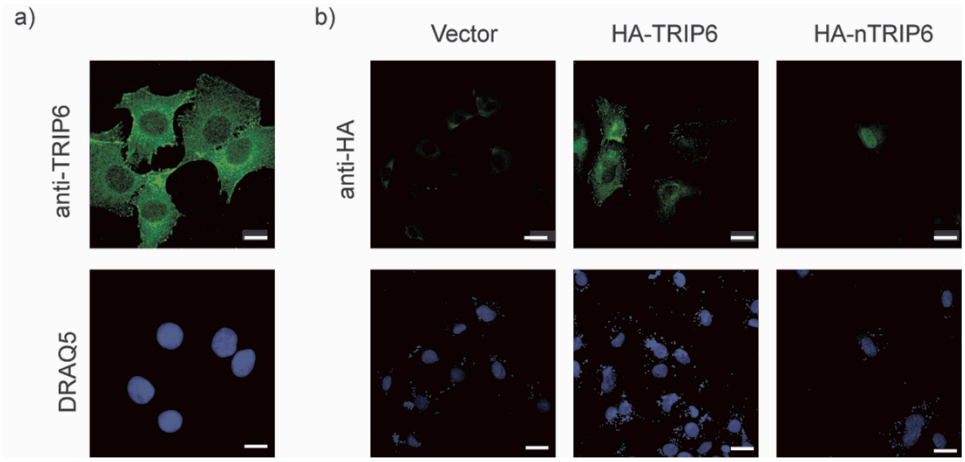

Figure 5 Subcellular localization of TRIP6 and nTRIP6 in myoblasts

a) C2C12 myoblasts were stained with an antibody recognizing both isoforms TRIP6 and nTRIP6. Nuclei were counterstained with DRAQ5. b) C2C12 cells were transfected with either an empty vector or an expression vector for TRIP6 or nTRIP6 fused to an HA-tag. Detection was performed using an anti-HA antibody and nuclei were counterstained with DRAQ5. (a-b) Representative confocal images are shown, scale bars: 10µm

This result suggests that TRIP6 might be shuttling from the cytosol to nucleus. To confirm this result, I expressed either an empty control vector or an expression vector for HA-tagged TRIP6. Overexpression of HA-TRIP6 led to an exclusive cytosolic staining. Thus, it is unlikely that the signal in the nucleus observed with the anti-TRIP6 antibody is TRIP6. (Fig. 5b). In our group, a shorter isoform of TRIP6 was identified which is exclusively present in the nucleus and therefore called nTRIP6 (Kassel et al., 2004). Indeed, ectopic expression of HA-nTRIP6 led to an exclusive nuclear staining (Fig. 5b). Taken together these results show that also in myoblasts, TRIP6 is present only in the cytosol, whereas nTRIP6 is located in the nucleus. Thus, if one of the isoforms acts as transcriptional co-regulator for MEF2C it must be nTRIP6.

To confirm that MEF2C interacts with nTRIP6 and not with TRIP6, I used the bimolecular fluorescent complementation (BiFC) assay, a protein-protein interaction assay based on the complementation of the fluorescent protein Venus (Hu et al., 2002). MEF2C was fused to the C-terminal half of Venus (VC) while TRIP6 and nTRIP6 were fused to the N-terminal half of Venus (VN). Immunofluorescence analysis of the constructs transfected in C2C12 cells showed that the fusion to VC or VN did not influence their subcellular localization (Fig. 6).

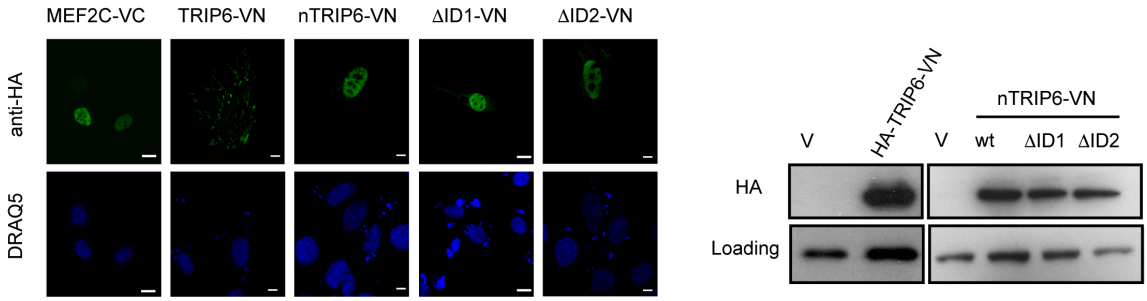

Figure 6 BiFC fusion Proteins are properly expressed and localized

C2C12 cells were transfected with expression vectors for HA-tagged versions of MEF2C fused to VC, TRIP6, nTRIP6, nTRIP6 lacking interaction domain 1 (ΔID1) or interaction domain 2 (ΔID2) fused to VN. Cells were then subjected to immunofluorescence analysis with an antibody against the HA tag and nuclei were counterstained with DRAQ5. Left panel: representative images are shown (scale bars: 10µm). Right panel: the same constructs were transfected into C2C12 cells and cell lysates were analyzed by Western Blotting using an anti-HA antibody and an anti-GR antibody as loading control.

Venus complementation was observed in only 7,3 % of the cells transfected with MEF2C-VC and TRIP6-VN, whereas it was observed in over 73% of the cells transfected with MEF2C-VC and nTRIP6-VN (Fig. 7). Furthermore, the BiFC signal was restricted to the nucleus. This result confirms that MEF2C interacts with nTRIP6 in the nucleus and not with TRIP6 in the cytosol. Preliminary work from the group showed that nTRIP6 interacts with MEF2C via its N-terminal pre-LIM region. Furthermore, two protein-protein interaction domains were identified in the N-terminal region of nTRIP6 (Diefenbacher et al., 2014). These domains are thus good candidates for mediating the interaction with MEF2C. These putative interaction domains will be further referred to as interaction domain 1 (ID1) and interaction domain 2 (ID2) that comprise the amino acids 175-187 and 253-265, respectively. To study the contribution of ID1 and ID2 to the interaction between MEF2C and nTRIP6, I designed deletion constructs lacking either of these domains and fused them to the N-terminal part of Venus. All deletion constructs were present in the nucleus and expressed at similar levels (Fig. 6). Deletion of either ID1 or ID2 led to a significant reduction in the interaction between nTRIP6 and MEF2C in the BiFC assay: only about 50% of the transfected cells showed Venus complementation (Fig. 7). This shows that both protein-protein interaction sites play a role in the binding of nTRIP6 to MEF2C.

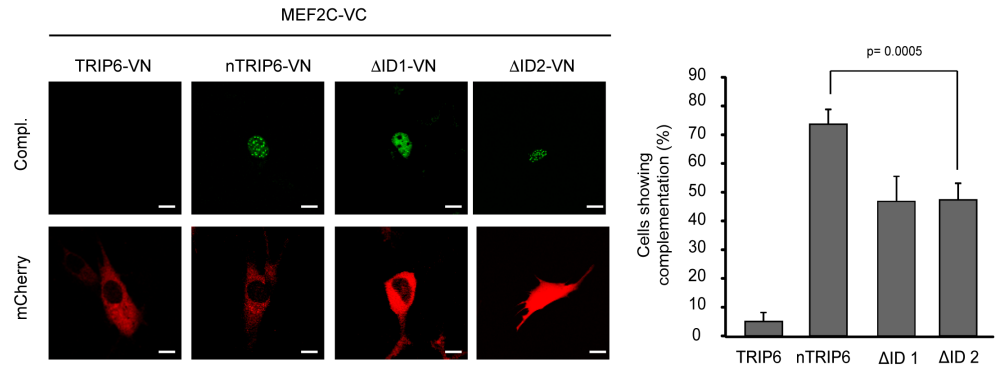

Figure 7 nTRIP6 interacts with MEF2C via distinct domains in the N-terminal region

C2C12 cells were co-transfected with expression vectors for MEF2C fused to the C-terminal half of Venus (VC) and either TRIP6 or nTRIP6, nTRIP6 lacking interaction domain 1 (ΔID1) or nTRIP6 lacking interaction domain 2 (ΔID2) fused to the N-terminal half of Venus (VN), together with mCherry fused to a nuclear export signal (NES) as a transfection control. Left panel, Venus complementation (Compl.) was imaged by confocal microscopy and representative images are shown (scale bars: 10µm). Right panel, the number of cells showing Venus complementation is presented as percentage of transfected, mCherry positive cells (mean ±SD of three independent experiments).

To confirm this result, I made use of genetically encoded blocking peptides that encode either ID1 or ID2 fused to mCherry and to a nuclear localization sequence to target them to the nucleus and therefore avoid any interference with TRIP6 in the cytosol. As negative controls I used a scrambled versions of the peptides (ID1c, ID2c) (Diefenbacher et al., 2014). The hypothesis was that if ID1 and ID2 are indeed involved in the interaction, the peptides would act as competitive inhibitors and interfere with the binding between nTRIP6 and MEF2C. C2C12 cells were transfected with nTRIP6-VN and MEF2C-VC and co-transfected with either the blocking peptides or the control peptides. The mCherry fluorescence signal confirmed that both peptides and their corresponding control peptides were properly localized to the nucleus (Fig. 8).

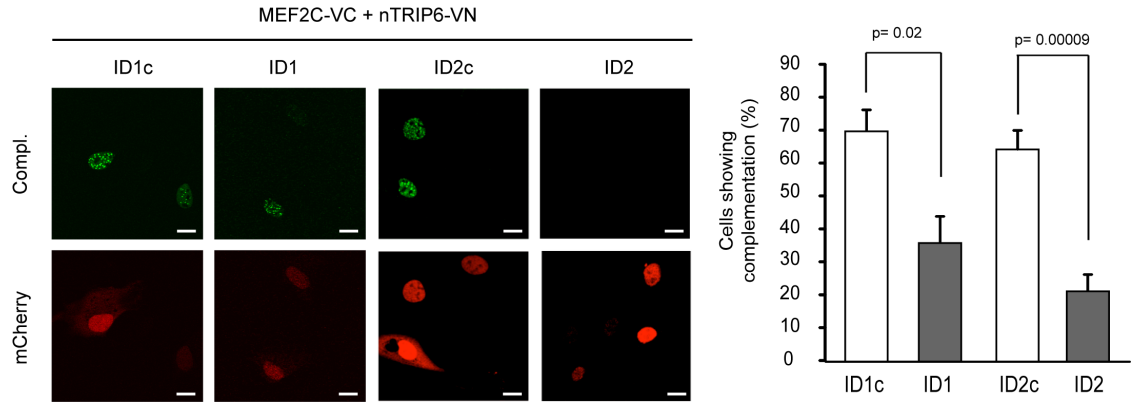

Figure 8 Blocking peptides inhibit the interaction between MEF2C and nTRIP6

C2C12 cells were co-transfected with MEF2-VC and nTRIP6-VN together with blocking peptides corresponding to either ID1 or ID2 fused to mCherry and a nuclear localization sequence (NLS). As controls scrambled versions of the blocking peptides were used (ID1c, ID2c). Left panel, Venus complementation (Compl.) was imaged by confocal microscopy and representative images are shown (Scale bars: 10µm). Right panel, the number of cells showing Venus complementation is presented as percentage of transfected, mCherry positive cells (mean ±SD of three independent experiments).

Transfection of the control peptides (ID1c, ID2c) had no influence on the interaction between nTRIP6 and MEF2C as 70% of the cells showed Venus complementation in the nucleus (Fig. 8), which is similar to the complementation observed between nTRIP6-VN and MEF2C-VC in the previous experiment (Fig. 7). In contrast, the number of cells showing complementation was reduced to 35% upon transfection with ID1 and to 20% upon transfection with ID2 (Fig. 8). This result confirms the

involvement of both N-terminal domains of nTRIP6 in its interaction with MEF2C. Taken together, nTRIP6, and not TRIP6 interacts with MEF2C in the nucleus of C2C12 myoblasts, the interaction is mediated via discrete protein-protein interaction domains in the N-terminus of nTRIP6 and blocking peptides can be used to specifically inhibit the interaction between nTRIP6 and MEF2C.

If nTRIP6 is a transcriptional co-regulator for MEF2C, it should be recruited to the MEF2C binding region of MEF2C target genes. To address this question, I performed Chromatin Immunoprecipitation (ChIP) assays in C2C12 cells. As MEF2C target genes I chose Myomesin 2 (Myom2) (Potthoff et al., 2007), Myoglobin (Mb) (Bassel-Duby et al., 1992), Troponin 1 type 2 (Tnni2) (Nakayama et al., 1996) and Desmin (Des) (Kuisk et al., 1996; Li and Capetanaki, 1994), which all have well characterized MEF2C binding sites. The ChIP assay shows that indeed nTRIP6 as well as MEF2C are present at the MEF2C binding site of these genes (Fig. 9a). A re-ChIP was performed to confirm that both proteins co-occupy these promoter regions. After chromatin precipitation with the antibody against TRIP6, the chromatin was eluted and subjected to a second precipitation using an antibody against MEF2C. The experiment showed that nTRIP6 and MEF2C were present together at the MEF2C binding site of the MEF2C target genes (Fig. 9b).

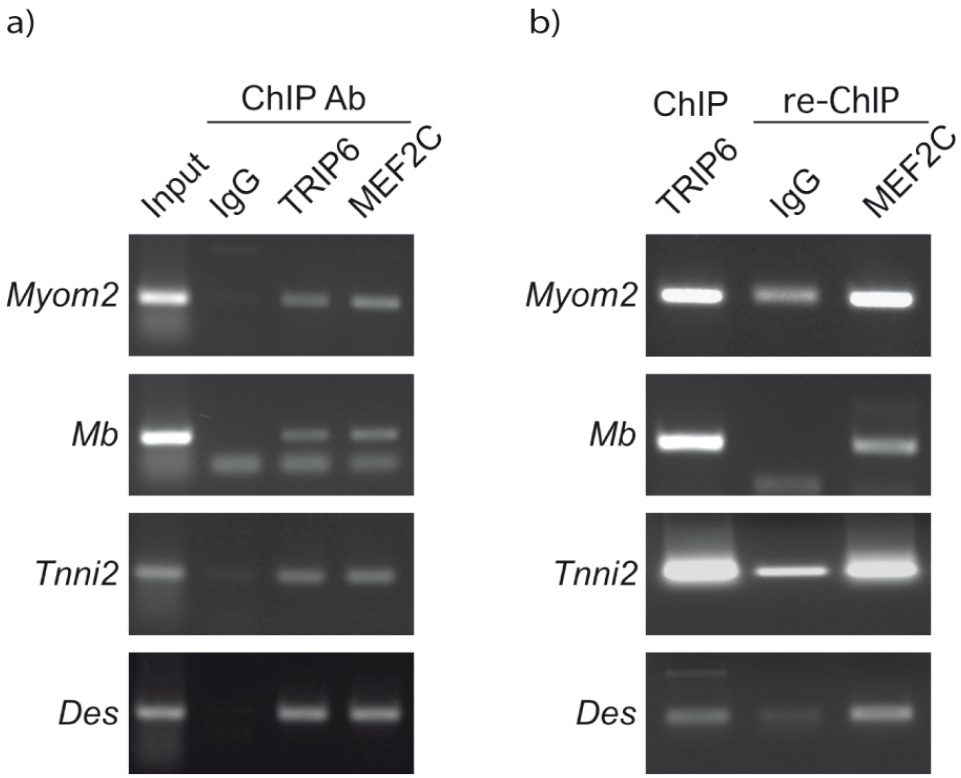

Figure 9 nTRIP6 and MEF2C co-occupy MEF2C-dependent promoters

a) Chromatin Immunoprecipitation (ChIP) was performed in C2C12 cells using the indicated antibodies (Ab). b) Chromatin immunoprecipitated with the anti-TRIP6 antibody was eluted and subjected to a re-ChIP using either the anti-MEF2C antibody or the corresponding isotype control antibody (IgG). (a-b) PCR was performed using primers flanking the MEF2C binding regions of the indicated genes. Gels from representative experiments are shown.

Taken together, nTRIP6 interacts with MEF2C and is recruited together with MEF2C to the regulatory regions of MEF2C target genes. Therefore, given the reported role for nTRIP6 as a transcriptional co-activator for GR, AP-1 and NF-κB (Diefenbacher et al., 2008, 2010, 2014) nTRIP6 might act as transcriptional co-activator for MEF2C. To address this question, I performed reporter gene assays in C2C12 cells using a MEF2C-dependent luciferase reporter construct. If nTRIP6 acts as coactivator for MEF2C, the loss of nTRIP6 should decrease MEF2C transcriptional activity. C2C12 cells were transfected with an siRNA targeting *Trip6* mRNA in order to knockdown nTRIP6 or with a nonspecific siRNA as a negative control.

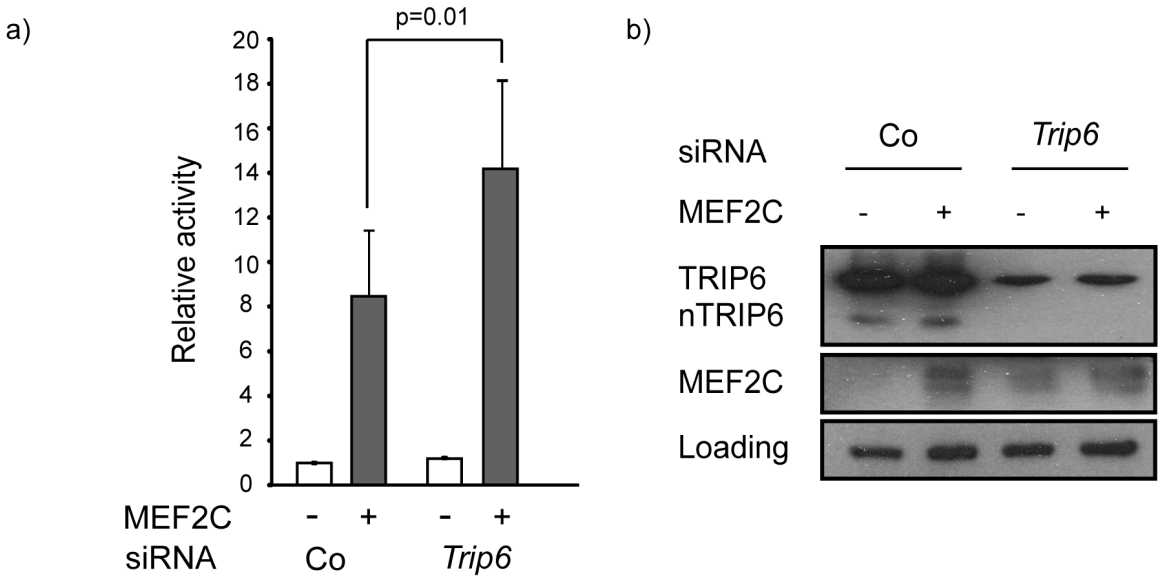

Figure 10 Silencing nTRIP6 increases MEF2C transcriptional activity

a) C2C12 cells were transfected with either an siRNA targeting *Trip6* mRNA or a control siRNA (Co). 24h post-transfection cells were transfected with a MEF2C-dependent luciferase reporter gene together with an expression vector for β-galactosidase for normalization and with an expression vector for MEF2C (+) or an empty vector (-). Normalized luciferase activity is plotted relative to the control siRNA, empty vector transfected cells (mean ± SD of three individuals experiments). b) After measurement of the luciferase activity, cell lysates were subjected to Western Blot analysis using the indicated antibodies, GR was used as loading control.

In the reporter gene assay, the MEF2C-dependent reporter gene was efficiently activated via transfection with MEF2C. When nTRIP6 was silenced the activity of the reporter construct was significantly increased, while the basal activity was not affected (Fig. 10a). Western Blot analysis confirmed that the specific siRNA efficiently knocked down nTRIP6 expression without affecting MEF2C expression (Fig. 10b). This experiment shows that surprisingly, nTRIP6 does not increase MEF2C transcriptional as should be the case for a transcriptional co-activator, but rather represses it. However, since TRIP6 expression was also knocked down I cannot exclude an indirect effect due to the loss of this isoforms. To confirm that nTRIP6 regulates MEF2C activity, I studied the effect of overexpressing nTRIP6 in the same reporter gene assay. MEF2C activity was repressed by nTRIP6 in a dose-dependent manner, while the expression of MEF2C was not affected (Fig. 11a).

Nevertheless, this still could be an indirect effect of overexpressed nTRIP6. Thus, I investigated whether the repression of MEF2C activity by nTRIP6 depends on the interaction between both proteins. nTRIP6 was not able to efficiently repress MEF2C activity when either the ID1 or ID2 interaction domains were deleted. (Fig.11a).

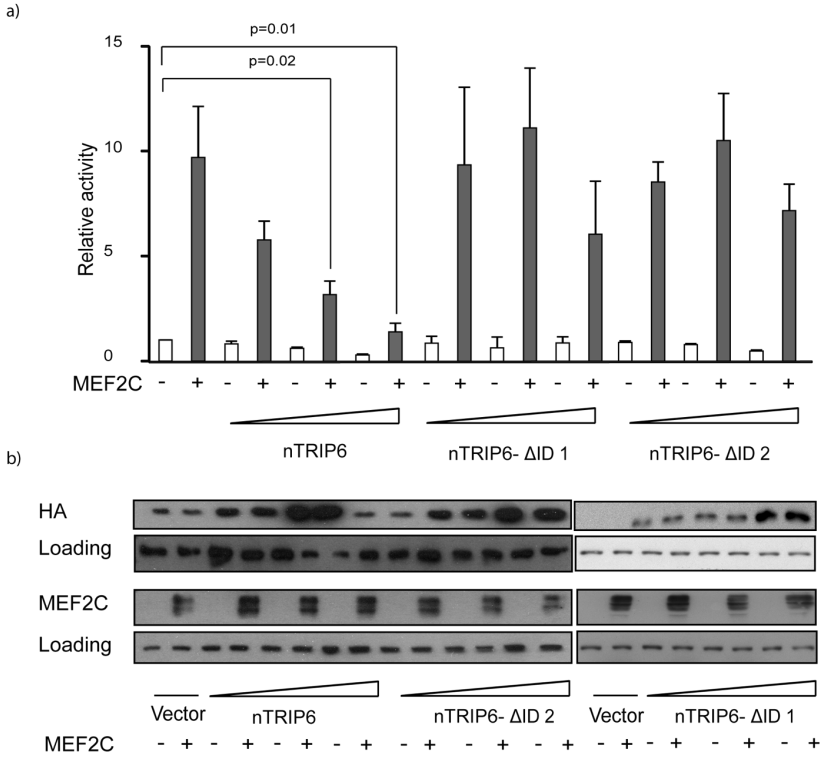

Figure 11 MEF2C repression depends on its interaction with nTRIP6

a) C2C12 cells were transfected with a MEF2C-dependent luciferase reporter construct, an expression vector for β-galactosidase for normalization, an expression vector for MEF2C (+) or empty vector (-), together with increasing amounts of either nTRIP6, nTRIP6 lacking the interaction domain 1 (ΔID1) or nTRIP6 lacking the interaction domain 2 (ΔID2). Normalized luciferase activity is plotted relative to the empty vector control. b) After measurement of the luciferase activity, cell lysates were subjected to Western Blot analysis using the indicated antibodies; GR was used as loading control.

This result shows that a direct binding of nTRIP6 via the interaction domains is necessary to repress MEF2C transcriptional activity, further pointing towards a direct effect of nTRIP6. In order to further confirm that the repression of MEF2C activity is carried out by the direct binding of nTRIP6 to MEF2C, the genetically encoded ID1 and ID2 peptides were used to block the interaction in the reporter gene assay.

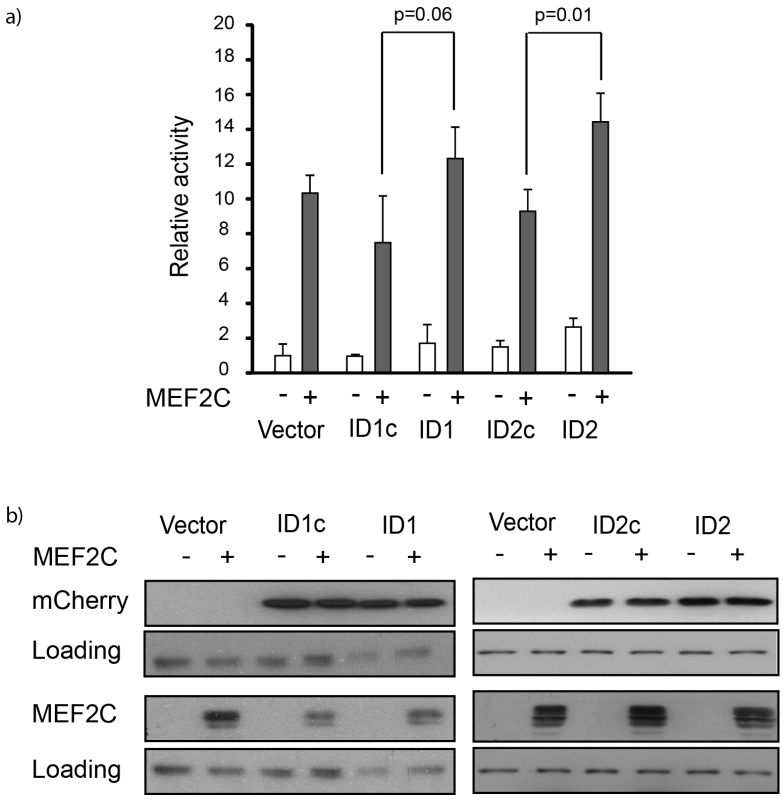

Figure 12 The blocking peptides increase MEF2C transcriptional activity

a) C2C12 cells were co-transfected with a MEF2C dependent luciferase reporter gene, an expression vector for β-galactosidase for normalization, either an expression vector for MEF2C (+) or an empty vector (-), together with either an empty vector or the mCherry-NLS fusion of either the ID1 peptide, its scrambled version (ID1c), the ID2 peptide or its scrambled version (ID2c). Normalized luciferase activity is plotted relative to the empty vector control (mean ±SD of three independent experiments). b) After measurement of the luciferase activity, cell lysates were subjected to Western Blot analysis using the indicated antibodies; GR was used as loading control.

While the control peptides did not have any influence on MEF2C activity, both blocking peptides increased the activity of MEF2C. However, ID2 was more efficient that ID1 (Fig. 12a). This result is in accordance with the previous observation that ID2 blocks the interaction between nTRIP6 and MEF2C more efficiently than ID1 (Fig. 8). For this reason, only ID2 was used for further experiments. Taken together, I demonstrated that nTRIP6 interacts with MEF2C and that both co-occupy the MEF2C response elements of MEF2C target genes. Moreover, nTRIP6 represses MEF2C transcriptional activity. Thus, nTRIP6 fulfills all criteria for beeing a transcriptional co-repressor for MEF2C.

3.2 nTRIP6 mediates the recruitment of HDAC5 to MEF2C target promoters

What could be the mechanism of nTRIP6 co-repressor function for MEF2C? nTRIP6 does not harbour any catalytic activity or functional domains classically found in transcriptional co-repressors. We have previously reported that nTRIP6 acts as transcriptional co-activator for other transcription factors by interacting via its LIM domains with and mediating the promoter recruitment of other co-regulators such as THRAP3, therebye acting as a bridging factor (Diefenbacher et al., 2014). A possible hypothesis is therefore that nTRIP6 recruits additional co-repressors to the promoter-bound MEF2C. Moreover, since nTRIP6 interacts with MEF2C via ist N-terminus, the LIM domains would still be available for recruiting further transcriptional co-regulators. Known co-repressors for MEF2C are class IIa Histone Deacetylases such as HDAC4 and HDAC5 (Lu et al., 2000a; McKinsey et al., 2000a, 2000b). Given the role for nTRIP6 as a bridging factor for other transcriptional co-regulators, I hypothesized that nTRIP6 mediates the recruitment of HDAC4 or HDAC5 to MEF2C-bound promoters. To investigate this hypothesis I first tested whether nTRIP6 interacts with HDAC4 and HDAC5 using the BiFC assay. Immunofluorescence staining showed that fusion of HDAC4 and HDAC5 to the N-terminal half of Venus did not hamper their expression or nuclear localization (Fig. 13a). The BiFC assay showed that nTRIP6 interacts with HDAC5 in the nucleus and that the interaction takes place via the LIM domains of nTRIP6. Interestingly nTRIP6 did not interact with HDAC4 (Fig. 13b). Given that the interaction between nTRIP6 and HDAC5 is mediated via the LIM domains and that nTRIP6 binds MEF2C via its N-terminal pre-LIM region, it is tempting to speculate that nTRIP6 is required for the interaction between MEF2C and HDAC5.

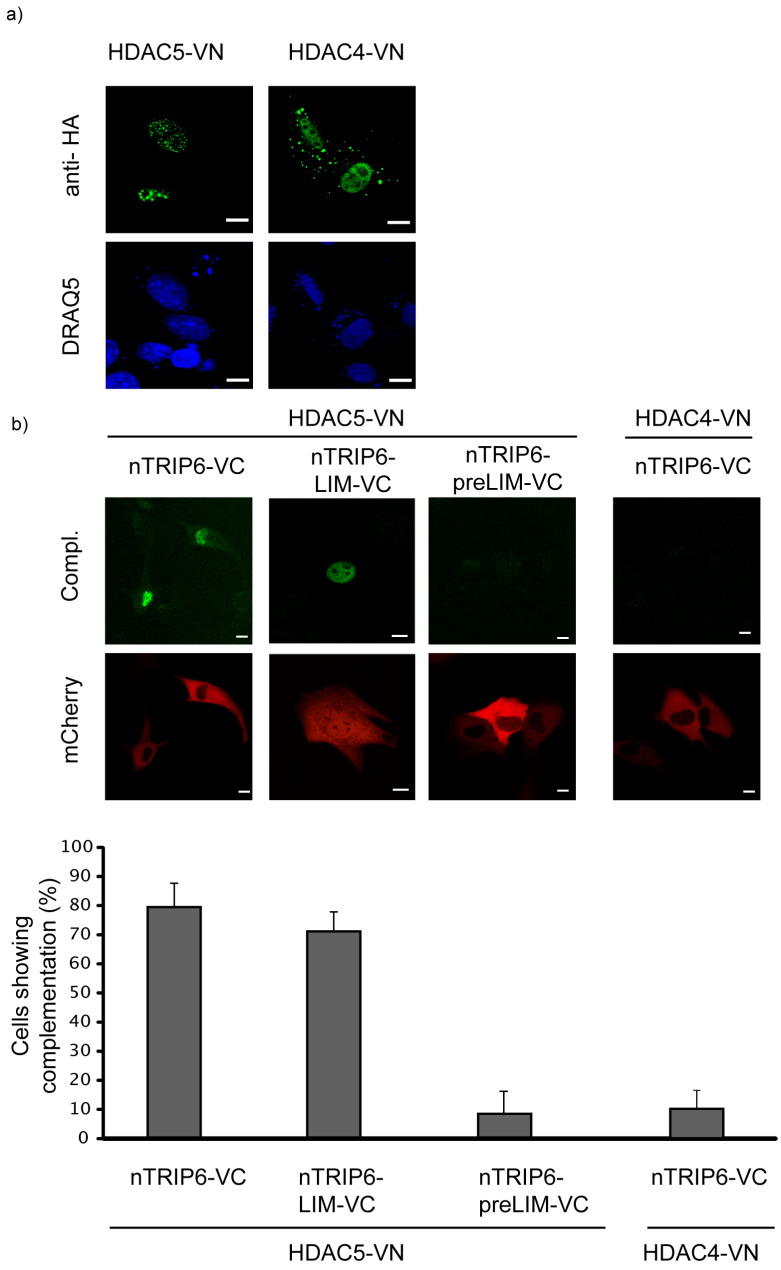

Figure 13 nTRIP6 interacts with HDAC5

a) C2C12 cells were transfected with HA-tagged versions of either HDAC5 or HDAC4 fused to the N-terminal half of Venus (VN) and subjected to immunofluorescence analysis using an antibody against the HA tag. Nuclei were counterstained with DRAQ5. Representative images are shown (Scale bars: 10µm). b) C2C12 cells were transfected with expression vectors for either HDAC5-VN or HDAC4-VN and either nTRIP6, only the LIM domains of nTRIP6 or the pre-LIM region of nTRIP6 fused to the C-terminus of Venus (VC). As a transfection control cells were transfected with an expression vector for mCherry fused to a nuclear export signal. Venus complementation (Compl.) was imaged by confocal microscopy and representative images are shown (Scale bars: 10µm). The number of cells showing Venus complementation is presented as the percentage of transfected, mCherry positive cells (mean ± SD of three independent experiments).

Thus, I tested whether the loss of nTRIP6 prevents the interaction between MEF2C and HDAC5. To do so nTRIP6 was silenced using the siRNA targeting *Trip6* mRNA, in C2C12 cells transfected with HDAC5 fused to the N-Terminal part of Venus and MEF2C fused to the C-terminal part of Venus (Fig. 14a). The siRNA efficiently knocked down nTRIP6 expression without affecting the expression of the BiFC constructs, as shown by Western Blot analysis (Fig. 14a). The loss of nTRIP6 led to a significant decrease in the number of transfected cells showing an interaction between MEF2C and HDAC5, as compared to the cells that were transfected with the control siRNA (Fig. 14a).

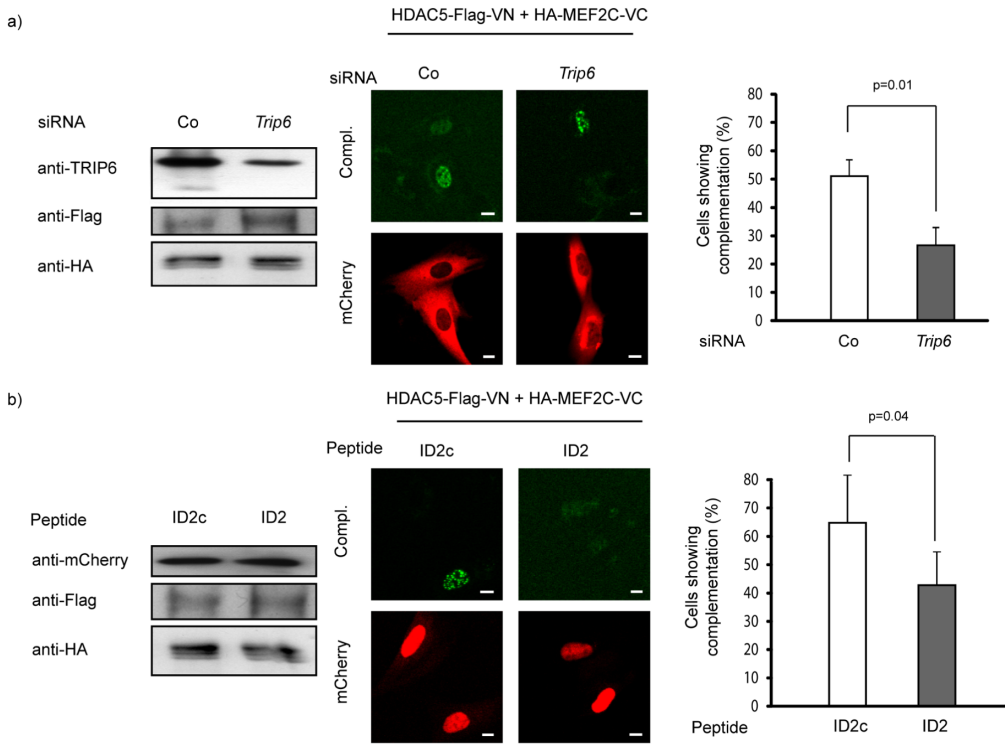

Figure 14 nTRIP6 mediates the interaction between MEF2C and HDAC5

a) C2C12 cells were transfected with an siRNA targeting the mRNA of *Trip6* or a control siRNA (Co). One day later the cells were transfected with Flag-tagged HDAC5 fused to VN and HA-tagged MEF2C fused to VC, together with mCherry fused to a nuclear export signal (NES) as a transfection control. b) C2C12 cells were co-transfected with HDAC5-Flag-VN and HA-MEF2C-VC together with the blocking peptide corresponding to ID2 or its scrambled version as a control (ID2c), both fused to mCherry and a nuclear localization sequence (NLS). a,b) Representative images are shown (Scale bars: 10µm). The number of cells showing Venus complementation is presented as a percentage of transfected, mCherry positive cells (mean ± SD of three independent experiments). Left panels: cell lysates from the experiments in (a-b) were subjected to Western blot analysis using the indicated antibodies.

Again, to exclude an indirect effect of the loss of TRIP6, the same BiFC experiment was performed in the presence of the ID2 peptide to block the interaction between endogenous nTRIP6 and MEF2C. Indeed, transfection of the blocking peptide significantly reduced the number of cells showing an interaction between HDAC5 and MEF2C (Fig. 14b). These results show that nTRIP6, interacting with MEF2C via its N-Terminal pre-LIM region and with HDAC5 via its LIM domains, acts as a bridging factor for the interaction between MEF2C and HDAC5. This is compatible with the hypothesis that nTRIP6 acts by mediating the recruitment of HDAC5 to the promoter-bound MEF2C. To address this hypothesis, I first investigated by ChIP the occupancy of the MEF2C binding sites of MEF2C target genes by HDAC5 and nTRIP6. Indeed, both proteins were present at the promoter of the MEF2C target genes Myomesin 2 (Myom2), Myoglobin (Mb), Troponin 1 type 2 (Tnni2) and Desmin (Des) (Fig.15a). Furthermore, re-ChIP using the antibody against HDAC5 after elution of chromatin precipitated with the anti-TRIP6 antibody confirmed that nTRIP6 and HDAC5 co-occupy the MEF2C binding site of these genes (Fig. 15b).

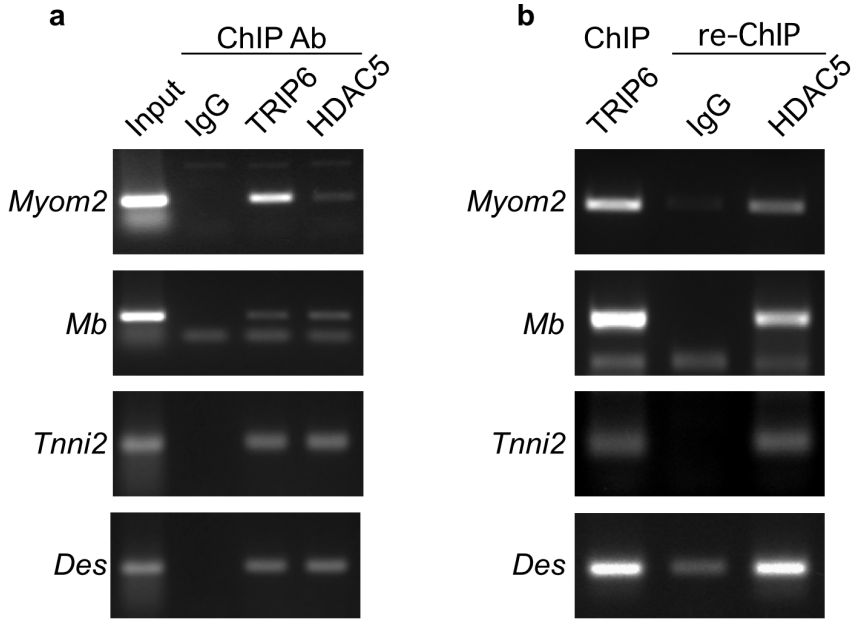

Figure 15 nTRIP6 and HDAC5 co-occupy MEF2C dependent promoters

a) Chromatin immunoprecipitation (ChIP) was performed in C2C12 cells using the indicated antibodies (Ab). b) Chromatin immunoprecipitated with the anti-TRIP6 antibody was eluted and subjected to a re-ChIP using either the anti-HDAC5 antibody or the corresponding isotype control antibody (IgG). (a-b) PCR was performed using primers flanking the MEF2C binding regions of the indicated genes. Gels from representative experiments are shown.

Finally, the putative bridging function of nTRIP6 was addressed. In the ChIP assay, the siRNA-mediated knockdown of nTRIP6 led to a reduction in the recruitment of HDAC5 to the MEF2C binding sites of MEF2C target genes (Fig. 16). Therefore, nTRIP6 acts as an adaptor co-regulator and recruits HDAC5 to the regulatory regions of MEF2C target genes.

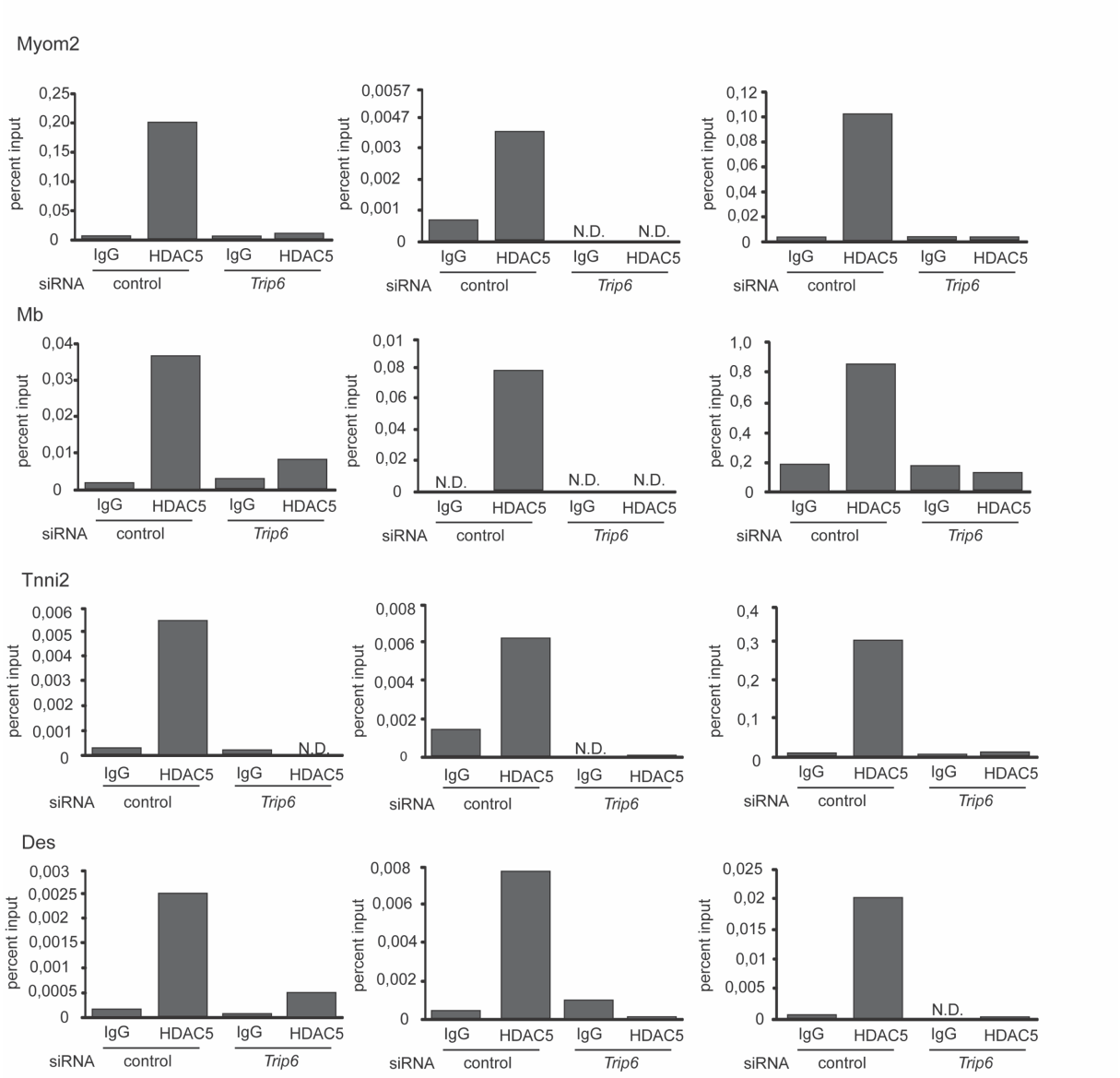

Figure 16 nTRIP6 mediates the recruitment of HDAC5 to MEF2C target promoters

C2C12 cells were transfected with either an siRNA targeting the mRNA of *Trip6* or a control siRNA (control). ChIP was performed using either an antibody against HDAC5 or an isotype control. Enrichments of the MEF2C binding regions of the indicated genes were determined by real Time PCR and plotted as percent of input. The results of 3 independent experiments are shown. N.D. : not detected.

To conclude this part, I have shown that nTRIP6 interacts with MEF2C via distinct domains in the pre-LIM region of nTRIP6. This interaction can be efficiently blocked by using a genetically encoded blocking peptide that competes with nTRIP6 for the binding to MEF2C. Moreover, nTRIP6 and MEF2C co-occupy the regulatory regions of MEF2C target genes leading to a repression of MEF2C activity. This repression is carried out by an nTRIP6-mediated recruitment of HDAC5 to the regulatory regions of MEF2C target genes. Thus, nTRIP6 acts as an adaptor-like transcriptional co-repressor for MEF2C.

3.3 Role of nTRIP6 in adult myogenesis

Given that nTRIP6 acts as a transcriptional co-repressor for MEF2C and that MEF2C drives the expression of differentiation genes during myogenesis, nTRIP6 might play a role in the regulation of differentiation and fusion. As first step in investigating a possible function for nTRIP6 in myogenesis, I studied its expression during the differentiation of the C2C12 myoblast cell line.

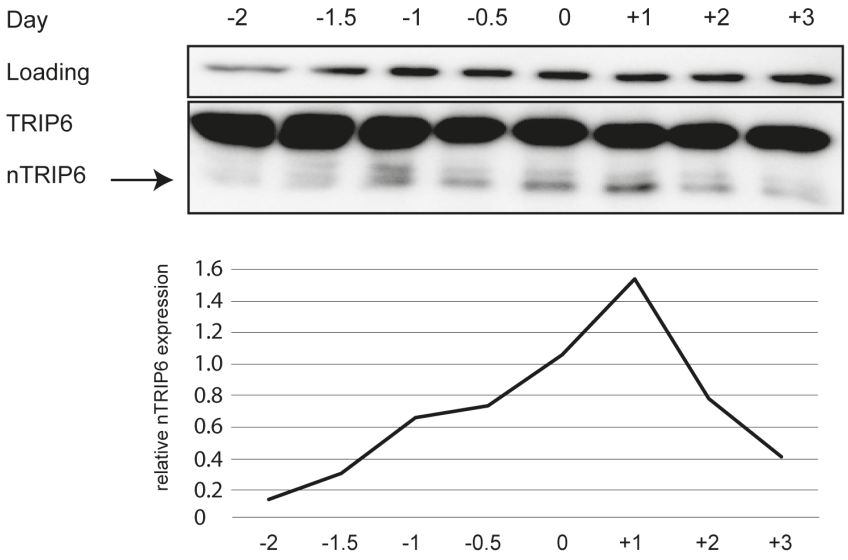

Figure 17 nTRIP6 expression is transiently increased prior to differentiation

C2C12 were seeded at low density and kept in proliferation medium for 2 days (day -2 till 0). At day 0 the medium was switched to low serum containing differentiation medium. Cells were harvested as indicated (day). Whole cell extracts were analysed by Western Blotting using an antibody against TRIP6 and nTRIP6. GR was used as a loading control. The relative expression of nTRIP6 and TRIP6 (ratio) was determined by quantifying the intensities of the respective bands on the blot (one representative blot shown)

While TRIP6 was expressed at constant levels during differentiation, nTRIP6 was barely detectable when the cells were at low density. nTRIP6 expression increased at the transition between late proliferation and early differentiation phase (from day-1 to +1) and decreases afterwards (Fig. 17). Interestingly the transient increase in nTRIP6 expression occurs when MEF2C activity has to be repressed in myoblasts (Liu et al., 2014). This observation suggests that nTRIP6 might play a role in the regulation of myoblast differentiation.

To investigate this hypothesis, I used the genetically encoded blocking peptide to block nTRIP6 function in myoblasts. As a readout, I analysed the expression of embryonic myosin heavy chain (MYH3), the first contractile protein that is expressed upon differentiation, and is classically used as a differentiation marker (Tomczak, 2003).

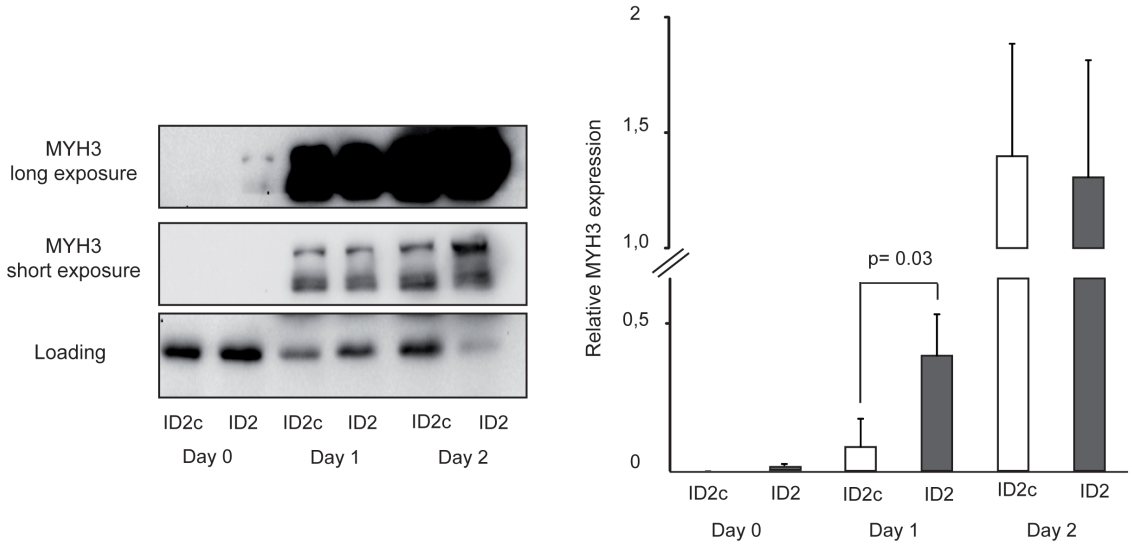

Figure 18 Blocking nTRIP6 function leads to premature differentiation

C2C12 cells were transfected with the ID2 blocking peptide or its scrambled version as a control (ID2c). After transfection, the cells were kept in proliferation medium for two days, then switched to differentiation medium (Day 0). Cells were harvested every day and whole cell lysate were analysed by Western Blotting using antibodies against MYH3 and GR as a loading control. Representative Western Blots are shown. The relative expression of MYH3, normalized to the expression of GR (ratio) was determined by quantifying the intensities of the respective bands on the blot (mean ± SD of three independent experiments).

In cells transfected with the control peptide, the expression of MYH3 started at day 1 after induction of differentiation (medium switch). However, when the blocking peptide was transfected myoblasts started expressing MYH3 one day earlier, before the cells were switched to differentiation medium (day =0). Moreover, at day

1 the expression of MYH3 was significantly increased as compared to the control (Fig. 18). Thus, in the presence of the nTRIP6 blocking peptide myoblasts differentiate too early. This result shows that nTRIP6 prevents the premature differentiation of myoblasts and thus plays a role in regulating the differentiation process.

I then tested whether blocking nTRIP6 function in myoblasts affects the fusion process. I used again the genetically encoded blocking peptide and determined the fusion index, i.e. the percentage of nuclei within fused fibres. In cells transfected with the control peptide, fusion of myoblasts started slowly at day 1 and rapidly increased to reach a fusion index of about 40% at day 3. However, in cells transfected with the blocking peptide the fusion index was significantly lower at both day 2 and day 3 (Fig. 19).

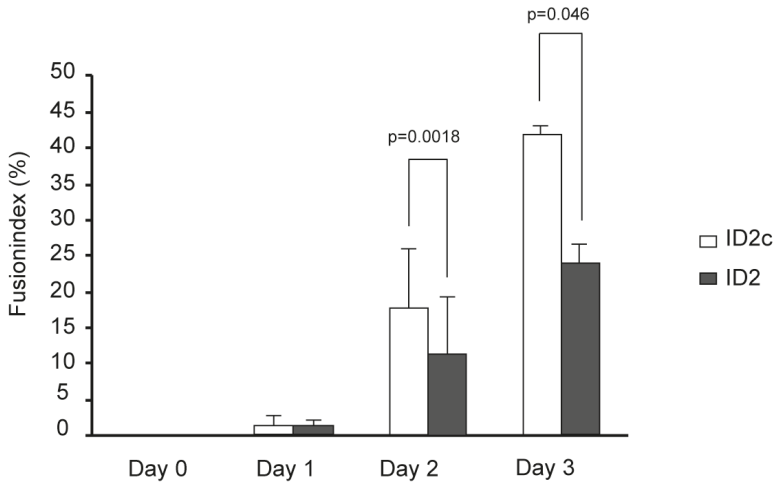

Figure 19 nTRIP6 function is required for proper fusion

C2C12 cells were transfected with the ID2 blocking peptide or its scrambled version as a control (ID2c). After transfection cells were kept in proliferation medium for two days. Then the medium was switched to differentiation medium (Day 0). During the fusion process cells were fixed every day and stained with an antibody against MYH3 to visualize the fibres, an anti-mCherry antibody and DRAQ5 to visualize the nuclei. Quantification was done by counting all the fibres within 1mm^2 and the number of nuclei within the fibres. Results are presented as the fusion index, i.e. the percentage of nuclei within fused fibres, and plotted as mean ± SD of three independent experiments.

These results show that although nTRIP6 blocks differentiation in myoblasts it is paradoxically required for proper fusion.

3.4 Role of nTRIP6 in muscle regeneration

In order to address the *in vivo* relevance of my findings showing a role for nTRIP6 in the regulation of myoblast differentiation *in vitro*, I investigated the muscle repair capacity of the conditional *Trip6* knockout mouse, in which the expression of TRIP6 and nTRIP6 is lost only in satellite cells. Briefly, animals in which the *Trip6* gene is flanked by LoxP sites (C57BL/6J-*Trip6*$^{fl/fl}$) were crossed with mice expressing the tamoxifen-inducible Cre recombinase (Cre-ER-T2) only in satellite cells (*Pax7*$^{CRE-ER-T2/wt}$). The resulting C57BL/6J-*Trip6*$^{fl/fl}$;*Pax7*$^{CRE-ER-T2/wt}$ animals were treated with tamoxifen to knockout the *Trip6* gene in satellite cells. I will refer to these animals as *Trip6*^{scKO}. The C57BL/6J-*Trip6*$^{fl/fl}$;*Pax7*$^{wt/wt}$ animals were used as controls and are referred to as *Trip6*$^{fl/fl}$.

As a first step I assessed the recombination efficiency in this mouse model. To do so single myofibres were isolated from the extensor digitorum longus muscle of tamoxifen-treated *Trip6*^{scKO} or *Trip6*$^{fl/fl}$ control animals and stained with an antibody against TRIP6/nTRIP6 and an antibody against PAX7 to identify satellite cells (Fig. 20).

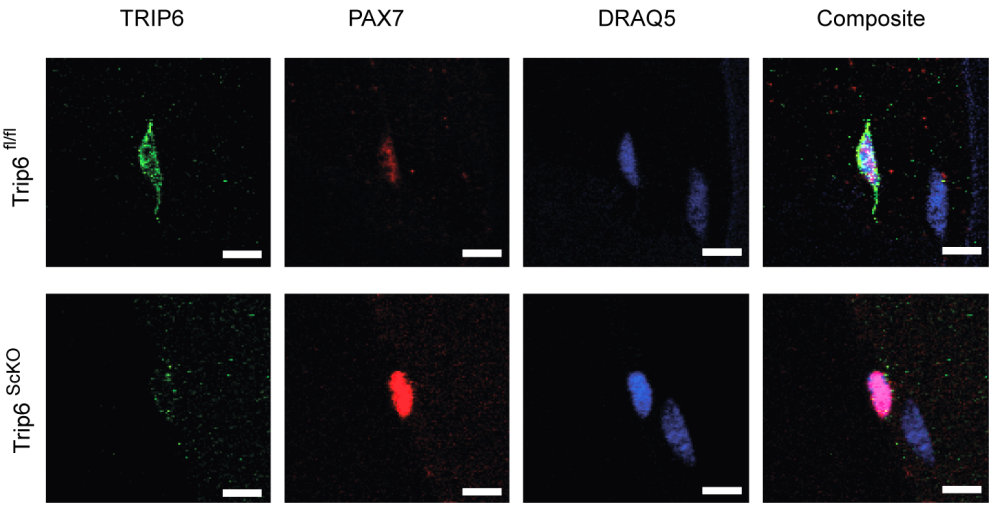

Figure 20 Efficient Trip6 gene knockout in satellite cells

Individual myofibres were isolated from extensor digitorum longus muscle of tamoxifen-treated C57BL/6J-*Trip6*$^{fl/fl}$;*Pax7*$^{CRE-ER-T2/wt}$ (Trip6^{ScKO}) mice or of C57BL/6J-*Trip6*$^{fl/fl}$;*Pax7*$^{wt/wt}$ (Trip6$^{fl/fl}$) littermate control animals and stained with antibodies against TRIP6/nTRIP6, PAX7 to identify satellite cells and DRAQ5 to visualize the nuclei. Representative images are shown (scale bar: 10μm).

Upon tamoxifen treatment, none of the PAX7 positive satellite cells showed any TRIP6 / nTRIP6 immunoreactivity (Fig. 20, quantification not shown), showing that the *Trip6* gene was efficiently knocked out in 100% of the satellite cells.
Muscle damage was induced by direct injection into the *M. soleus* of Notexin, a toxin from the venom of the Australian tiger snake *Notechis scutatus*. This toxin induces the complete degeneration of the muscle by destruction of the myofibres, without damaging the satellite cells (Dixon and Harris, 1996). Animals were sacrificed after 7, 10, 14 and 28 days of regeneration and the size of the regenerating myofibre was quantified as an index of regeneration (Fig. 21).

In *Trip6*$^{fl/fl}$ (wt) animals, the size of regenerating myofibres had already reached about 50% of the fibre size in the non-damaged contralateral muscle after 7 days of regeneration. This size steadily increased until day 28 post-injury. Importantly, there was no significant difference in the size of the fibres in undamaged muscles of *Trip6*$^{fl/fl}$ and *Trip6*^{scKO} mice (minimum Feret's diameters of 26.6±3.2 µm and 26.5±3.2 µm, respectively, mean ± SD, n=20). In the regenerating muscle of the *Trip6*^{scKO} animals, after 7 days of regeneration the size of the regenerating fibres was larger than in the control animals, suggesting an accelerated regeneration process. This difference was no more observable at day 10 post-injury. However, at later time points, the size of the *Trip6*$^{fl/fl}$ fibres significantly increases from day 10 to day 14, which was not the case for the *Trip6*^{scKO} mice. Even after 28 days the fibres from the *Trip6*^{scKO} mice did not reach the size of the *Trip6*$^{fl/fl}$ mice (Fig. 21).

This impaired regeneration in the *Trip6*^{scKO} mouse suggests defects in either the differentiation or the fusion of myoblasts. To directly address this question, I quantified the number of MYOD-expressing mono-nucleated myogenic cells, i.e. myoblasts and myocytes during regeneration.

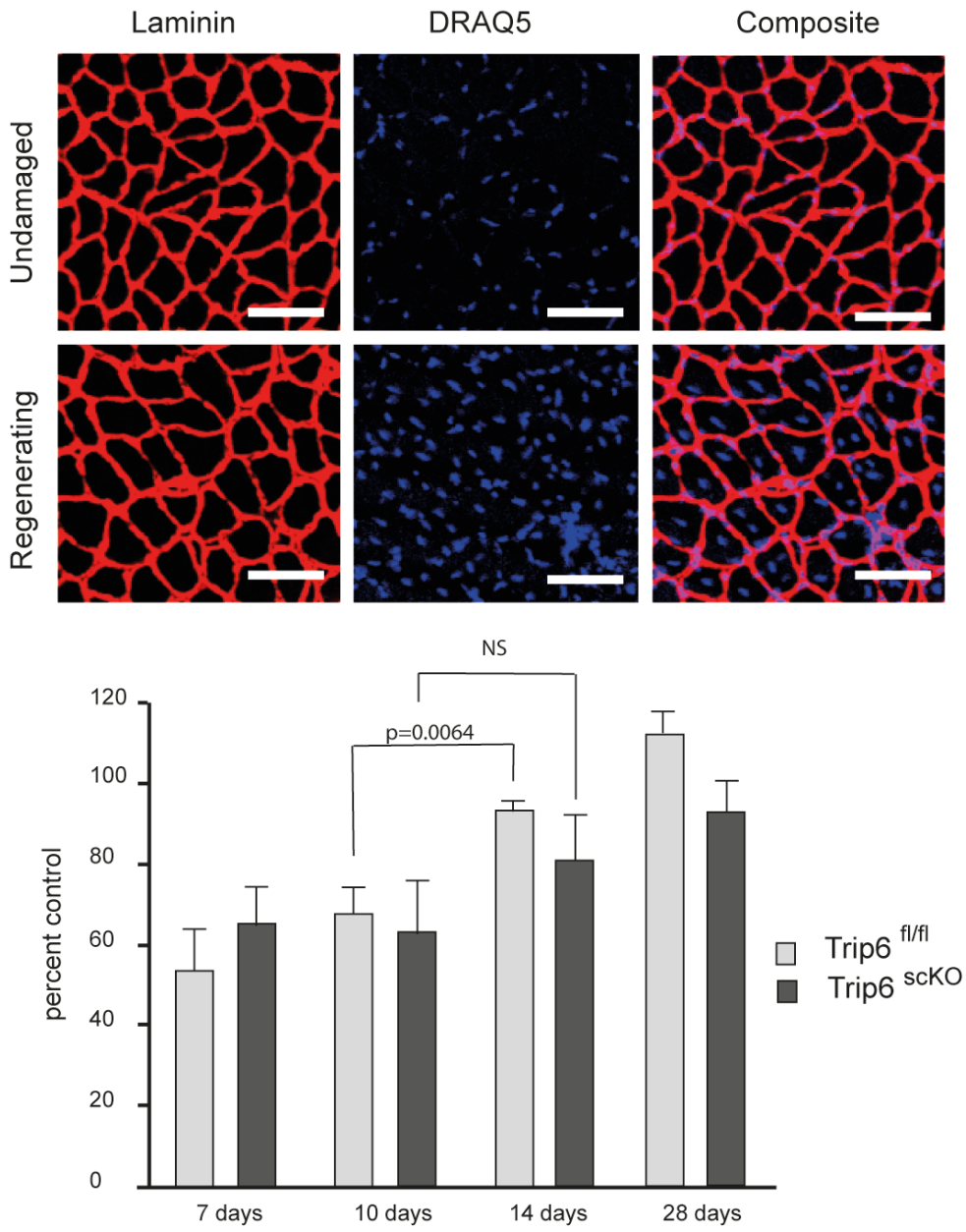

Figure 21 Muscle regeneration is impaired in satellite cell specific Trip6 knockout mice

Cross-sections of the undamaged and regenerating soleus muscle from either *Trip6*^{scKO} or *Trip6*$^{fl/fl}$ littermate control animals were stained with an antibody against laminin to delineate the myofibres and with DRAQ5 to visualize the nuclei. Regenerating myofibres were identified by their centralized nuclei. a) Representative images are shown from *Trip6*$^{fl/fl}$ mice after 14d of regeneration (scale bar: 100μm). b) Myofibre size was measured by automated segmentation of the individual myofibres and calculation of the minimum Feret's diameter using ImageJ. The myofibre size is plotted as percentage of the undamaged contralateral leg (mean ± S.D. of groups of n=5). Statistical analysis was performed according to 2.2.6 of the methods section.

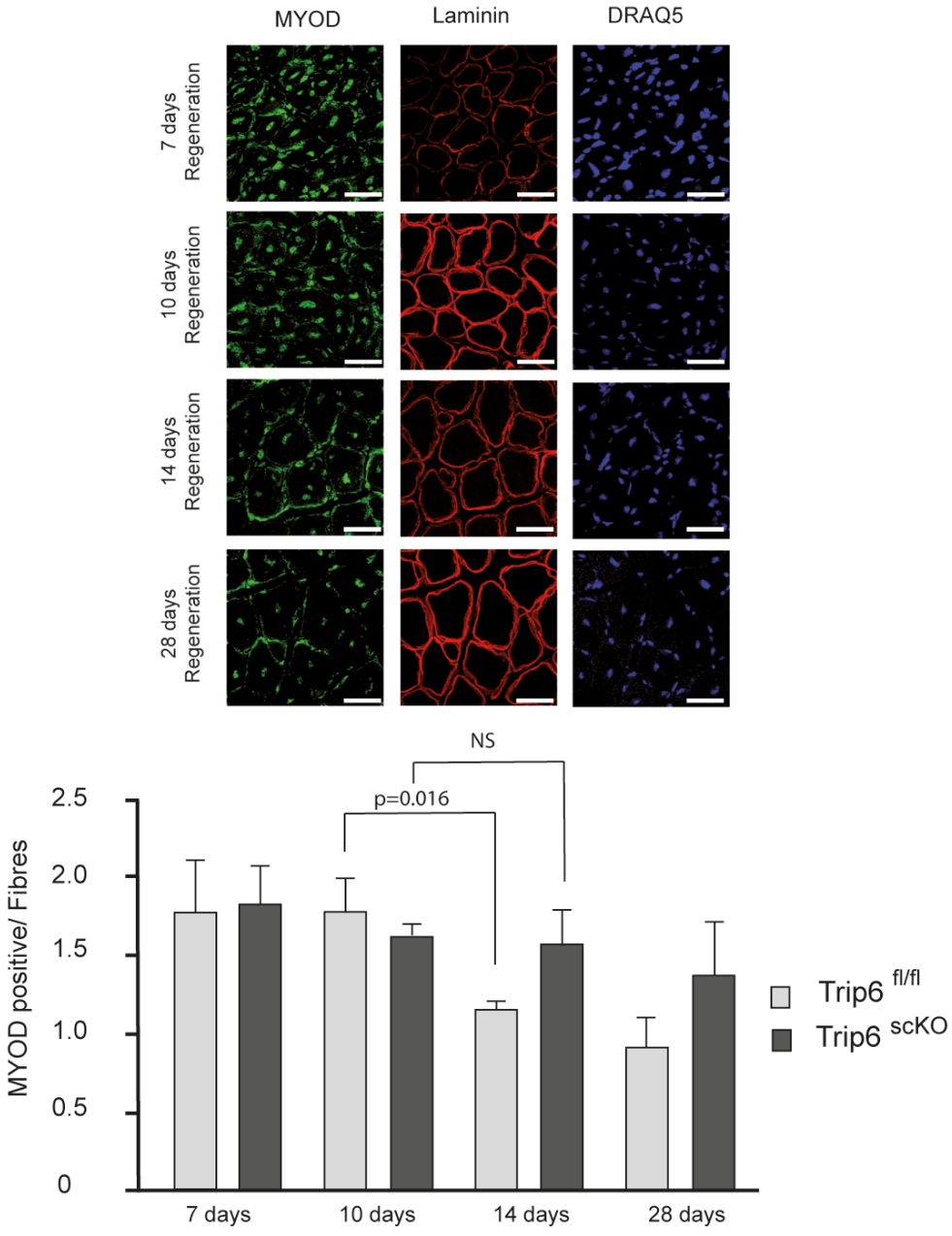

Figure 22 The number of unfused myoblasts/myocytes does not decrease during muscle regeneration in satellite cell specific Trip6 knockout mice

Cross sections of soleus muscle from either *Trip6*^{scKO} or *Trip6*$^{fl/fl}$ littermate control mice treated with Notexin were stained after 7, 10, 14 and 28 days of regeneration with an antibody against MYOD to visualize myoblasts and myocytes, an antibody against Laminin to delineate the myofibres with and DRAQ5 to visualize the nuclei. a) Representative images are shown from *Trip6*$^{fl/fl}$ mice (scale bar: 50μm). b) Quantification was performed by counting all MYOD positive mononucleated cells and normalizing this number to the number of fibres on the section (mean ± S.D. of groups of n=5). Statistical analysis was performed according to 2.2.6 of the methods section.

In the regenerating soleus muscle of the *Trip6*$^{fl/fl}$ control animals, the number of MYOD positive cells that were not fused started to significantly decrease after 10 days of regeneration (Fig. 22). In the muscle of *Trip6*^{scKO} mice, at the first day of regeneration I observed, 7 and 10 days post-injury, the number of non-fused MYOD positive cells was similar to that in the muscles of the control mice. However, from day 10 to day 14 this number of MYOD positive cells decreases significantly in the *Trip6*$^{fl/fl}$ animals but not in the *Trip6*^{scKO} mice (Fig. 22). Therefore, the impaired regeneration in terms of fibre size upon knocking out the *Trip6* gene in satellite cells appears to be due to an impaired differentiation and fusion of myoblasts.

4. Discussion

In this work, I have uncovered a novel function for nTRIP6, the short isoform of the focal adhesion LIM domain protein TRIP6: it acts as a co-repressor for the myogenic transcription factor MEF2C in proliferating myoblasts. Furthermore, during adult myogenesis nTRIP6 expression is transiently increased directly before differentiation starts. Blocking its function results in a premature expression of late differentiation genes combined with a reduced myocyte fusion. Finally, in a model of muscle regeneration in the *Trip6* knockout mouse, regenerated myofibres had a smaller size, and the regenerating muscle showed a higher number of unfused myogenic cells than in the control animals.

4.1 nTRIP6 acts as transcriptional co-repressor for MEF2C

In the first part of this thesis, I report a that nTRIP6 acts as a co-repressor for the transcription factor MEF2C in proliferating myoblasts. Transcriptional co-repressor can be defined as factors which do not directly bind DNA but are recruited to the regulatory regions of target genes through a direct or indirect interaction with DNA-binding transcription factors, and which participate in the repression of transcription. I showed that nTRIP6 interacts with MEF2C via its N-terminal pre-LIM region, is recruited together with MEF2C to the regulatory regions of MEF2C target genes, and represses MEF2C transcriptional activity. Thus nTRIP6 fulfils all criteria for being a transcriptional co-repressor for MEF2C. Although TRIP6 also harbours the domains in the nTRIP6 pre-LIM region that are responsible for the interaction with MEF2C, TRIP6 does not interact with MEF2C in the nucleus of proliferating myoblasts. This observation is in contrast to the reported findings in which other focal adhesion LIM domain proteins of the Zyxin family exert nuclear functions by shuttling between the focal adhesion sites and the nucleus upon, for example, changes related to regulation of focal adhesion (Cattaruzza et al., 2004; Mori et al., 2012; Yoshigi et al., 2005). In the nucleus, these focal adhesion LIM domain proteins can interact with a variety of nuclear proteins and transcription factors and influence their transcriptional activity (Wang and Gilmore, 2003). My results confirm that although TRIP6 belongs to the Zyxin family, its nuclear function

is not depending on signalling that induces TRIP6 translocation to the nucleus. Like its co-activator function for AP-1, NF-κB and GR (Diefenbacher et al., 2008, 2010, 2014; Kassel et al., 2004), TRIP6 co-repressor function for MEF2C is exerted by the smaller isoform nTRIP6 which is exclusively present in the nucleus (Kassel et al., 2004).

My results show that nTRIP6 acts as an adaptor co-repressor for MEF2C in proliferating myoblasts by mediating the promoter recruitment of HDAC5, but, surprisingly, not that of HDAC4, another related class IIa HDAC. The activity of both these known co-repressor of MEF2C in myoblasts (Lu et al., 2000a; McKinsey et al., 2000a) is mainly regulated via a common mechanism targeting their subcellular localization. HDAC4 and 5 contain a NES as well as an NLS. Upon phosphorylation of serine residues near the NLS sequence, the protein 14-3-3 binds HDAC4 and 5 and masks the NLS. This leads to a nuclear export of both proteins (McKinsey et al., 2000b). My observation that nTRIP6 interacts with HDAC5 in the nucleus but not with HDAC4 suggests the existence of different mechanisms regulating HDAC4 and HDAC5 function in myoblasts. One may speculate that the interaction with nTRIP6 in the nucleus may be a mechanism to differentially regulate the nuclear export of both proteins: the interaction with nTRIP6 might be an active mechanism to retain HDAC5 in the nucleus. Such a difference in the regulation of HDAC4 and HDAC5 has already been observed in another context: in fast type myofibres, low frequency electrical stimulation induces the nuclear export of HDAC4 but not of HDAC5 (Liu et al., 2005). My observations further illustrate the complexity of MEF2C regulation by class IIa HDACs in myoblasts.

By mediating the interaction between MEF2C and HDAC5 at the regulatory regions of MEF2C target genes, nTRIP6 acts as an adaptor co-repressor. A similar function of nTRIP6 has previously been reported in the regulation of AP-1, NF-κB and GR transcriptional activity where nTRIP6 acts as an adaptor co-activator by mediating the recruitment of THRAP3 (Diefenbacher et al., 2014). Thus nTRIP6 is able to act as either a co-activator or a co-repressor, depending on the transcription factor it interacts with. Other members of the Zyxin and Paxillin families of LIM domain proteins have been shown to be able to act as both co-activator and co-repressor.

For example HIC-5 is a co-activator for the androgen receptor (AR) and GR, and at the same time acts as co-repressor for SMAD and LEF/TCF (Ghogomu et al., 2006; Wang et al., 2005).Similarly, AJUBA acts as a co-repressor for SNAIL (Hou et al., 2008) and as a co-activator for PPARγ (Li et al., 2016). How can the same adaptor protein mediate the recruitment of either a co-activator or a co-repressor? When nTRIP6 co-activates AP-1 the interaction with the transcription factor occurs via its LIM domains (Kassel et al., 2004). However, when nTRIP6 co-represses MEF2C, the interaction with the transcription factor occurs via the pre-LIM region and not via the LIM domains. Could this difference in the mode of interaction with the regulated transcription factor be responsible for whether it acts as a co-activator or a co-repressor? Interestingly, when AJUBA acts as an adaptor co-repressor for SNAIL, it interacts with SNAIL via its LIM domains (Hou et al., 2008) and recruits the co-repressor PRMT5 via its pre-LIM region. However, when it co-activates PPARγ, it interacts with this transcription factor via its pre-LIM region and recruits the co-activator p300/CBP via its LIM domains (Li et al., 2016). Thus, it seems tempting to speculate that the way by which focal adhesion LIM domain proteins interact with transcription factors may affect the ability of the LIM domains to interact with either a co-activator or a co-repressor. Another hypothesis would be that nTRIP6 recruits either co-activators or co-repressors depending on their availability or level in the nucleus. Indeed, MEF2C repression via HDAC5 takes place during myoblast proliferation, a time point where HDAC5 is present at high levels in the nucleus (Lu et al., 2000a; McKinsey et al., 2000b). Thus, nTRIP6 most likely interacts with HDAC5 because of its high levels in the nucleus. This hypothesis is strengthened by the role of nTRIP6 in the repressive crosstalk between AP-1 and GR (Jonat et al., 1990; Lucibello et al., 1990; Schüle and Evans, 1991). In the absence of GR in the nucleus, nTRIP6 recruits the co-activator THRAP3 to AP-1-bound promoters. However, upon stimulation with glucocorticoids, GR translocates into the nucleus, interacts with nTRIP6, displaces THRAP3 and thus represses AP-1 activity (Diefenbacher et al., 2008, 2010; Kassel et al., 2004). Therefore, the availability of GR in the nucleus appears to be the prime determinant of the ability of nTRIP6 to act as an adaptor co-repressor for AP-1. These examples suggest that indeed nTRIP6 acts as co-activator or co-repressor depending on the availability of other co-regulators in the nucleus.

During myogenesis, MEF2C drives the expression of genes that are involved in late differentiation, fusion and maturation (Hinits and Hughes, 2007; Potthoff et al., 2007). However it is already expressed in proliferating myoblasts (Liu et al., 2014; Mokalled et al., 2012) and its activity must therefore be repressed. This repression is at least in part carried out by HDAC5 (Lu et al., 2000b; McKinsey et al., 2000b). My results showing that in proliferating myoblasts nTRIP6 co-represses MEF2C via HDAC5 suggest that nTRIP6 might participate in the inhibition of differentiation in proliferating myoblasts.

4.2 Role of nTRIP6 in adult myogenesis

As a first hint to a putative role for nTRIP6 during myogenesis, I have shown that its expression is regulated. nTRIP6 is expressed at very low levels at the beginning of myoblast proliferation. Its expression then strongly increases at the transition between late proliferation and early differentiation. nTRIP6 expression then decreases back to basal levels during late differentiation and fusion. Interestingly, nTRIP6 levels are at the highest when MEF2C is already expressed in myoblasts and its activity has to be repressed, and decrease when MEF2C activity is required for late differentiation. Given the function of nTRIP6 as a transcriptional co-repressor for MEF2C, the dynamics of nTRIP6 expression during myogenesis is totally compatible with a role for nTRIP6 in the transient repression of MEF2C activity during late proliferation and early differentiation. Indeed, the loss of nTRIP6 function led to the premature expression of late differentiation genes such as MYH3 (this work), Myomesin 2, Troponin I2 and Myoglobin (Kemler et al., 2016) during the early differentiation phase. Since the expression of some of these structural genes such as Myomesin 2 and Troponin I2 is driven by MEF2C, the repression of their premature expression by nTRIP6 might be directly related to the co-repressor function of nTRIP6 for MEF2C. However, MYH3 is not a direct MEF2C target gene but rather a MYOD target gene (Tapscott, 2005). During myogenesis MEF2C alone is not sufficient to drive myogenic differentiation. MEF2C cooperates with MYOD and amplifies its transcriptional activity, and drives the expression of other MRFs, such as myogenin, that promote late differentiation (Buchberger et al., 1994; Molkentin et al., 1995; Ridgeway et al., 2000). It is tempting to speculate that

nTRIP6 regulates not only MEF2C but also MYOD activity by an indirect mechanism. For example, MEF2C is known to directly interact with MYOD at the promoter of MYOD target genes, acting as a co-regulator of MYOD (Badodi et al., 2015; Molkentin et al., 1995; Sartorelli et al., 1997). It is therefore conceivable that MEF2C could mediate the recruitment of nTRIP6 to MYOD-bound promoters, leading to a transcriptional repression. Consequently, the loss of nTRIP6 function could also induce the premature activation of MYOD target genes such as MYH3. Interestingly, MYOD also drives the expression of p21 (Guo et al., 1995) and other cell cycle regulators that induce myoblasts to exit from the cell cycle (Andrés and Walsh, 1996; Guo et al., 1995; Walsh and Perlman, 1997). If nTRIP6 indeed controls their expression during the late proliferation phase, the loss of nTRIP6 function might deregulate the coordinated transition between proliferation arrest and early differentiation. Such a mechanism might be responsible for the increased number of MYOD positive cells that I observed *in vivo* in the regenerating muscles when the *trip6* gene was knocked out in satellite cells.

In addition, nTRIP6 might not only play a role in preventing premature differentiation by acting as a co-repressor for MEF2C, and putatively indirectly for MYOD. Indeed, nTRIP6 has been reported to act as a co-activator for AP-1 and NF-κB (Diefenbacher et al., 2008, 2010, 2014; Kassel et al., 2004). Interestingly, both these transcription factors are known to promote proliferation of myoblasts and inhibit their differentiation by repressing the activity of MRFs (Lassar et al., 1989; Li et al., 1992). Thus it is tempting to speculate that nTRIP6 might not only be able to block differentiation in myoblasts by co-repressing transcription factors that stimulate differentiation but also by co-activating transcription factors that repress differentiation. Taken together my results strongly suggest that the transient increase in nTRIP6 expression is essential to prevent the premature differentiation of proliferating myoblasts.

Interestingly, although nTRIP6 has an anti-differentiation effect in proliferating myoblasts, at least in part by blocking MEF2C activity, my experiments also showed that, paradoxically, blocking nTRIP6 function in myoblast leads to an impaired myocyte fusion. The same effect was observed *in vivo* where

regenerating myofibres showed a smaller size when the *trip6* gene was knocked out in satellite cells. However, in this model of regeneration both nTRIP6 and TRIP6, the long cytosolic isoform are knocked out and a combined effect due to the loss of both isoforms cannot be excluded. TRIP6, which is mainly located at focal adhesion sites, has been reported to be involved in the migration of cells (Bai et al., 2007; Lai et al., 2005, 2007). Satellite cells have been shown to be highly motile and migrate along the muscle fibres (Siegel et al., 2009). Thus, it is possible that when TRIP6 is lost, satellite cells fail to migrate to the location of the damage and consequently cannot contribute to the regeneration process. Furthermore, TRIP6 also plays a role in cytoskeleton dynamics (Lin and Lin, 2011; Sanz-Rodriguez et al., 2004; Xu et al., 2004). During late myocyte differentiation and fusion, a massive re-arrangement of the cytoskeleton is needed to align the cells in a parallel fashion and to let them fuse. Immediately before fusion, a dense actin wall forms between the fusing cells that is supposed to give the correct membrane rigidity for the final fusion process (Duan and Gallagher, 2009). Then small gaps appear in the actin wall that form the fusion pore (Robertson et al., 1990). Therefore, the loss of TRIP6 in myocytes might interfere with these cytoskeletal rearrangement processes, leading to a defect in the fusion process during muscle regeneration *in vivo*. This has to be further investigated. Nevertheless, my *in vitro* data rather hint towards a role for nTRIP6 in fusion. Indeed, the blocking peptide I used specifically blocks the function of nTRIP6 in the nucleus, without influencing TRIP6 action in the cytosol. Yet the question remains: how can a nuclear protein such as nTRIP6 influence myocyte fusion? A direct effect of nTRIP6 at sites of myocyte fusion or a direct involvement in processes such as cytoskeletal rearrangement can be excluded because of the exclusive nuclear localization of nTRIP6. One possibility would be that nTRIP6 might be required for the expression of genes needed to drive fusion. However, the dynamics of nTRIP6 expression during myogenesis does not seem compatible with such an effect. Indeed, my results show that when myocyte fusion starts nTRIP6 expression has already decreased and is very low. Therefore, it seems rather unlikely that nTRIP6 is involved in the fusion process directly. This hints towards a rather indirect role in which nTRIP6 is required at earlier stages to then allow for a proper fusion at later stages. One logical hypothesis is that this effect of nTRIP6 on fusion is related to its differentiation inhibitory function. Indeed, the premature activation of MEF2C

due to the loss of nTRIP6 leads to the expression of proteins of the contractile apparatus already during late proliferation (this study and Kemler et. al. 2016). The premature presence of these huge protein complexes might interfere with the rearrangement of the cytoskeleton or with the formation of fusion pores. This might result in a failure of these myocytes to fuse either with each other or with existing fibres. This hypothesis would also explain my observation that in *trip6* knockout mice many MYOD positive cells stay unfused during the regeneration process, even after 28 days of regeneration. These unfused cells are most likely the reason for the smaller size of the regenerated myofibres, since the growth of muscle fibres is depending on the fusion of myocytes with the existing fibres (Granata et al., 1998). However, it is still unclear at which stage of the differentiation process the unfused MYOD positive cells are. Indeed, MYOD starts to be expressed in satellite cells upon activation, then its expression strongly increases during proliferation and differentiation and is reduced during late differentiation and fusion (Bentzinger et al., 2012). Therefore, it is possible that the MYOD expressing cells are stuck in the proliferation process, or that these cells have already undergone differentiation but fail to fuse with the regenerating myofibres. Additional experiments have to be performed in order to dissect these possibilities.

4.3 Conclusion

In this work I have uncovered a novel molecular function for nTRIP6, which was so far known as a transcriptional co-activator, as a transcriptional co-repressor for MEF2C in myoblasts. This work also illustrates the complexity of the regulation of MEF2C activity during myogenesis. Furthermore, my work revealed a novel role for nTRIP6 as a regulator of adult myogenesis. In conclusion, I propose a model (Fig. 23) whereby a transient increase in nTRIP6 expression during late proliferation and early differentiation of myoblasts prevents the premature induction of late differentiation genes and structural proteins, most likely through the co-repressor function of nTRIP6 for MEF2C. This anti-differentiation function of nTRIP6 at these early time points appears to be required to allow a proper myocyte fusion at later time points. Thus, the temporal regulation of nTRIP6 expression modulates the dynamics of adult myogenesis by coordinating the transition between proliferation and differentiation.

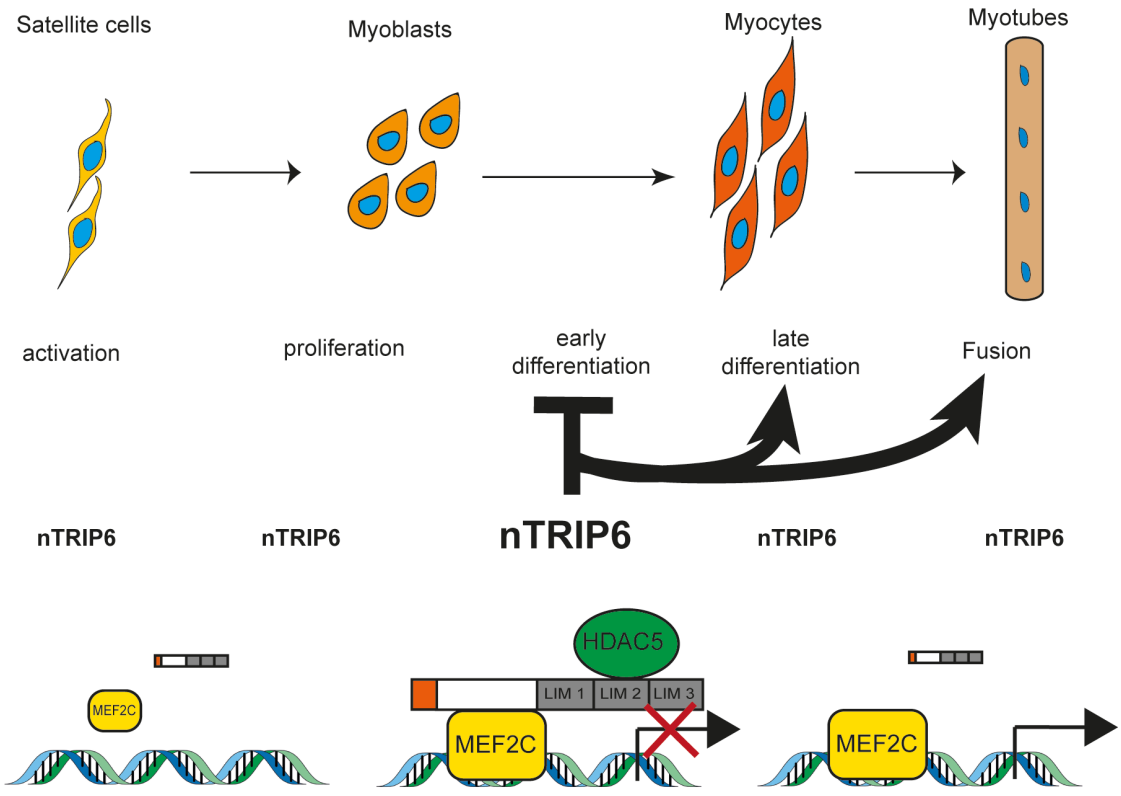

Figure 23 Proposed model of nTRIP6 role in the regulation of adult myogenesis

During the early stages of myoblast proliferation, the expression of nTRIP6 is very low. It then transiently increases during late proliferation, a time point at which MEF2C is already expressed but should be repressed. At this critical transition between proliferation and early differentiation, nTRIP6, expressed at high levels, acts as a transcriptional co-repressor for MEF2C by mediating the recruitment of HDAC5 to promoter-bound MEF2C. This transient repression of MEF2C activity prevents premature differentiation of myoblasts. nTRIP6 then decreases back to basal levels, allowing MEF2C to drive late differentiation and fusion. Importantly, the transient, early increase in nTRIP6 expression appears critical to enable a proper differentiation and fusion at later time points.

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