The dynamic function of nTRIP6 in the regulation of myogenesis

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Zita Gonda

I. Zusammenfassung

Die Skelettmuskulatur verfügt über eine bemerkenswerte Regenerationsfähigkeit, die auf adulte Stammzellen, die sogenannten Satellitenzellen zurückzuführen ist. Entsteht ein Schaden an den Muskeln, werden Satellitenzellen aktiviert, welche proliferierende Vorläuferzellen, die Myoblasten ausbilden. Diese Myoblasten verlassen den Zellzyklus und differenzieren zu Myozyten, die anschließend miteinander fusionieren, um neue unreife Myofasern zu bilden. Dieser präzise koordinierte zelluläre Vorgang wird als postnatale Myogenese bezeichnet, und wird durch ein Netzwerk von Transkriptionsfaktoren reguliert. Im ersten Teil meiner Arbeit habe ich die Rolle des transkriptionellen Koregulators nTRIP6, welcher die kurze nukleäre Isoform des LIM Domänen Proteins TRIP6 ist, bei der Regulation der Myogenese untersucht. Durch die Verwendung von C2C12 Myoblastenzellen als *in vitro* Modell für die Myogenese konnte ich zeigen, dass die Expression von nTRIP6 beim Übergang zwischen Proliferation und Differenzierung von Myoblasten vorübergehend ansteigt. Diese temporäre Zunahme an nTRIP6 erfolgte durch eine erhöhte Translationsinitiation an einem internen AUG (AUG2) innerhalb der *Trip6* mRNA. Eine selektive Blockierung der Translationsinitiation von nTRIP6 an AUG2 führte zu einer beschleunigten frühen und verzögerten späten Myozytendifferenzierung, sowie zu einer beeinträchtigten Myozytenfusion. Durch den frühen vorübergehenden Anstieg der Translation von nTRIP6 wird die vorzeitige Differenzierung der Myoblasten verhindert, um in späteren Stadien eine korrekte Differenzierung und Fusion zu ermöglichen.

Ein Vorgang, der zur Erhaltung des Stammzellenpools der Skelettmuskulatur beiträgt, ist die Bildung von sogenannten Reservezellen. Hierbei verlässt eine Subpopulation von Myoblasten den Zellzyklus, entgeht dem Differenzierungsprogramm, und kehrt durch einen wenig erforschten Mechanismus in den Ruhezustand zurück. Angesichts der inhibierten Differenzierung durch nTRIP6 stelle ich die Hypothese auf, dass nTRIP6 eine Rolle bei der Bildung von Reservezellen spielt. Ich beobachtete, dass eine Subpopulation von Myoblasten während der späten Differenzierung und Fusion von Myozyten eine hohe Expressionsrate von nTRIP6 aufrechterhielt. Nach vollendeter Fusion wurden Reservezellen durch die Expression von PAX7 identifiziert, einem Marker für ruhende Zellen. Diese Zellen wiesen eine hohe nTRIP6 Expressionsrate auf.

Darüber hinaus führte die erzwungene Expression von nTRIP6 in proliferierenden Myoblasten zu einer erhöhten Expression von PAX7. Aufgrund dieser Ergebnisse nehme ich an, dass nTRIP6 eine Rolle bei der Bildung von Reservezellen spielt.

Zusammenfassend möchte ich vorschlagen, dass die inhibierte Differenzierung durch nTRIP6 eine doppelte Rolle bei der Regulierung der postnatalen Myogenese spielt. Es steuert einerseits in der frühen Myogenese den zeitlichen Beginn der Differenzierung, trägt jedoch später der Widerherstellung des ruhenden Stammzellpools bei.

II. Abstract

Skeletal muscle has a remarkable regenerative capacity, which relies on adult stem cells, the satellite cells. Upon injury, satellite cells become active and give rise to proliferating progenitor cells, the myoblasts. These myoblasts then become cell cycle arrested and differentiate into committed myocytes, which then fuse together to form new immature myofibres. This highly orchestrated cellular process is called postnatal myogenesis and is regulated by a network of transcription factors. In the first part of my work, I investigated the role of the transcriptional co-regulator nTRIP6, the short nuclear isoform of the LIM domain protein TRIP6, in the regulation of myogenesis. Using the C2C12 myoblast cell line as an *in vitro* model for myogenesis, I showed that nTRIP6 expression transiently increased at the transition between myoblast proliferation and differentiation. This transient increase in nTRIP6 levels occurred via an increased translation initiation at an internal AUG (AUG2) within the *Trip6* mRNA. Selectively blocking nTRIP6 translation initiation at AUG2 resulted in an accelerated early and delayed late myocyte differentiation, as well as in an impaired myocyte fusion. Thus, the early transient increase in nTRIP6 translation prevents premature myoblast differentiation to allow a proper differentiation and fusion at later stages.

One mechanism that contributes to the replenishment of the skeletal muscle stem cell pool is the formation of so-called reserve cells. Here, a subpopulation of myoblasts exits the cell cycle, escapes the differentiation program and returns to quiescence through a poorly understood mechanism. Given the anti-differentiation function of nTRIP6, I hypothesized a role for nTRIP6 in reserve cell formation. I observed that a subpopulation of myoblasts maintained high levels of nTRIP6 during late myocyte differentiation and fusion. After fusion was complete, reserve cells, as identified by their expression of the quiescence marker PAX7, expressed high levels of nTRIP6. Furthermore, enforced expression of high levels of nTRIP6 in proliferating myoblasts resulted in an increased PAX7 expression. Together, these results strongly suggest that nTRIP6 plays a role in reserve cell formation.

In conclusion, I propose that through its anti-differentiation function, nTRIP6 plays a dual role in the control of postnatal myogenesis: an early role for the temporal control of the onset of differentiation and a late role for the replenishment of the quiescent cell pool.

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V. Abbreviations

AUG	Adenine-uracil-guanine translation initiation codon
CPP	Cell penetrating peptide
iuORF	Internal upstream open reading frame
MRF	Myogenic regulatory factor
MYOG	Myogenin
nTRIP6	Nuclear thyroid hormone receptor interactor protein 6
ORF	Open reading frame
PBS-/-	Phosphate-buffered saline without Ca^{++} and Mg^{++}
PNA	Peptide nucleic acid
TRIP6	Thyroid hormone receptor interactor protein 6
uORF	Upstream open reading frames

1. Introduction

1.1 Skeletal muscle tissue

The skeletal muscle takes up approximately 40% of the total body mass in the human body. It has essential functions from both mechanical and metabolic perspectives. On the one hand, it generates force, maintains posture and produces movement. On the other hand, it largely contributes to basal energy metabolism. It produces heat maintaining core temperature and consumes most of the available oxygen and nutrients during physical activity (Frontera and Ochala, 2015). Furthermore, it serves as a storage for amino acids which are needed in the absence of nutrient intake to replenish the amino acid reservoir of the blood needed for protein synthesis in other tissues, such as skin, brain and heart (Biolo et al., 1995). 50-75% of all proteins in the body belong to the skeletal muscle tissue, which is therefore responsible for 30-50% of the overall protein turnover (Frontera and Ochala, 2015). At inadequate nutrient supply, skeletal muscle alone is able to cover the amino acid demand of the hepatic glucose neogenesis thereby keeping the plasma glucose concentration constant (Felig, 1973).

The skeletal muscle consists of bundles of cylindrical muscle cells, referred to as myofibres. A bundle of myofibres, called a fasciculus, is enveloped by a layer of connective tissue, the perimysium. Myofibres are multinucleated (myonuclei) syncytial cells covered by a connective tissue called the endomysium, which contains the capillaries and nerves necessary for oxygen and nutrient supply as well as muscle control. Each myofibre consists of thousands of embedded myofibrils, which are packed within myofilaments. These myofilaments are arranged together in a highly ordered manner resulting in repeating contractile units, the sarcomeres (Frontera and Ochala, 2015). The main contractile proteins of the sarcomeres are myosin and actin. The thick filament, myosin, is the main molecular motor which burns ATP. Its head moves back and forth while attached to the thin filament, actin, thus generating muscle contraction (Rayment et al., 1993). Contraction regulatory proteins such as tropomyosin and troponin make sure of an adequate muscle function, i.e. contraction activation and kinetics (Frontera and Ochala, 2015).

Skeletal muscle is a highly plastic and adaptable tissue. Physiological stimuli, such as nutritional status, physical activity, aging, disease or injury influence the balance between protein synthesis and degradation resulting in the regulation of muscle mass. Muscle loss is not only associated with a decreased mechanical capacity, but also with an impaired ability of the body to respond to stress and chronic illnesses (Frontera and Ochala, 2015). Thus, skeletal muscle needs constant repair or regeneration.

1.1.1 Skeletal muscle repair and regeneration

Under normal conditions the turnover of adult skeletal muscle is a relatively slow process, with about 1-2% myonuclei being replaced per week (Schmalbruch and Lewis, 2000). However, large numbers of new myotubes can be generated in 3-4 days after severe muscle damage, endowing skeletal muscle with a remarkable capability for repair and regeneration (Whalen et al., 1990). When focal damage occurs to a specific area, it is sufficient for the muscle to undergo repair. For example, small membrane damage can be repaired by the recruitment of intracellular vesicles to reseal the disrupted cell membrane (Steinhardt et al., 1994). In contrast, severe trauma that makes it necessary for a full contractile myofibre to be replaced, leads to a tightly controlled degeneration and regeneration process of the muscle. These muscle injuries disrupt the integrity of myofibre plasma membrane and basal lamina causing muscle degradation and myonecrosis. (Grounds, 2014; Yang and Hu, 2018). The discontinuity of the muscle membranes leads to the activation of immune responses promoting the inflammatory phase of muscle regeneration. Aside hematoma formation, mast cells and neutrophils infiltrate the necrotic tissue to remove the damaged myofibres. Then, via cytokine secretion they recruit more immune cells such as macrophages (Ciciliot and Schiaffino, 2010). A first population of macrophages helps to clean up the debris at the site of the lesion by phagocytosis. Later, a second population of macrophages is involved by promoting the start of an anti-inflammatory phase and by activating the muscle stem cells, the satellite cells, which set off muscle regeneration (Tidball and Wehling-Henricks, 2007). This suppression of inflammation is essential for

proper tissue recovery (Vannella and Wynn, 2017). Upon activation, satellite cells start to proliferate and migrate to the site of injury, where they initiate the regeneration process (Goetsch et al., 2013).

1.1.2 Postnatal myogenesis

The cellular process involved in skeletal muscle regeneration, referred to as postnatal myogenesis, is initiated by resident stem cells, the satellite cells. This name originates from their position between the basal lamina and the sarcolemma of the myofibre (Mauro, 1961). Satellite cells, which express the transcription factors PAX3 and PAX7, are derived from mononuclear somite muscle precursor cells. During embryogenesis, these cells do not differentiate into myofibres but instead form a pool of quiescent cells. As quiescent cells, they are characterized by a small nucleus along with a condensed chromatin and are in a cell cycle-arrested, transcriptionally inactive state (Schultz et al., 1978). Satellite cells play a crucial role under physiological conditions in postnatal muscle growth, as well as under pathological conditions in regeneration (Pallafacchina et al., 2013; Relaix et al., 2006). During skeletal muscle regeneration, upon muscle injury a satellite cell becomes activated, enters the cell cycle, and starts to extensively proliferate giving rise to transit amplifying cells, the myoblasts (Figure 1). These cells undergo several rounds of divisions before they exit the cell cycle and enter the differentiation phase as committed progenitor cells, the myocytes. Myocytes then either fuse to already existing myofibres or they fuse together to form a new immature syncytial myofibre, a myotube. A hallmark for regenerated myotubes is their centrally located myonuclei. During terminal maturation, the myonuclei of the myotubes move to the periphery, and eventually they become new contractile muscle cells, the myofibres (Relaix and Zammit, 2012).

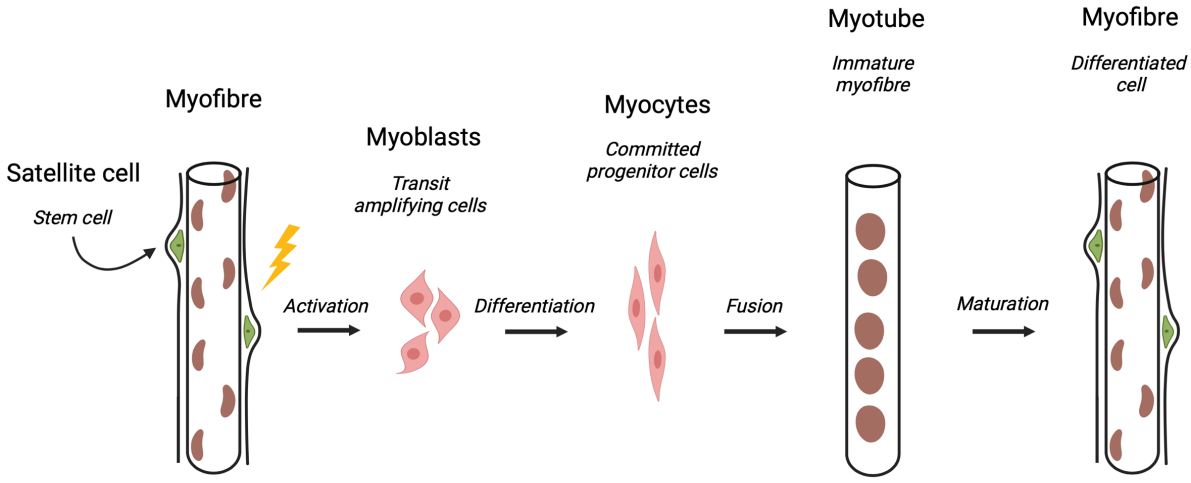

Figure 1. Mechanism of adult myogenesis. Upon muscle injury satellite cells become active, exit quiescence, and start to proliferate as transit amplifying cells, the myoblasts. After several rounds of divisions, these differentiate into committed progenitor cells, the myocytes. Myocytes then either fuse to already existing myofibres or they fuse together to form a new immature myofibre, the myotube, which after a maturation process becomes a differentiated muscle cell, the myofibre.

1.1.3 Myogenic transcription factors

Postnatal myogenesis is a complex multistep process requiring a precise regulation. A panel of myogenic transcription factors are responsible for the tight temporal control, assuring the progression through activation, proliferation and differentiation (Figure 2). Three families of transcription factors are predominantly involved in the regulation of postnatal myogenesis: paired-box-containing (PAX) transcription factors, myogenic regulatory factors (MRFs) and members of the MADS-box containing transcription factor family, in particular the myocyte enhancer factor-2 (MEF2) transcription factor.

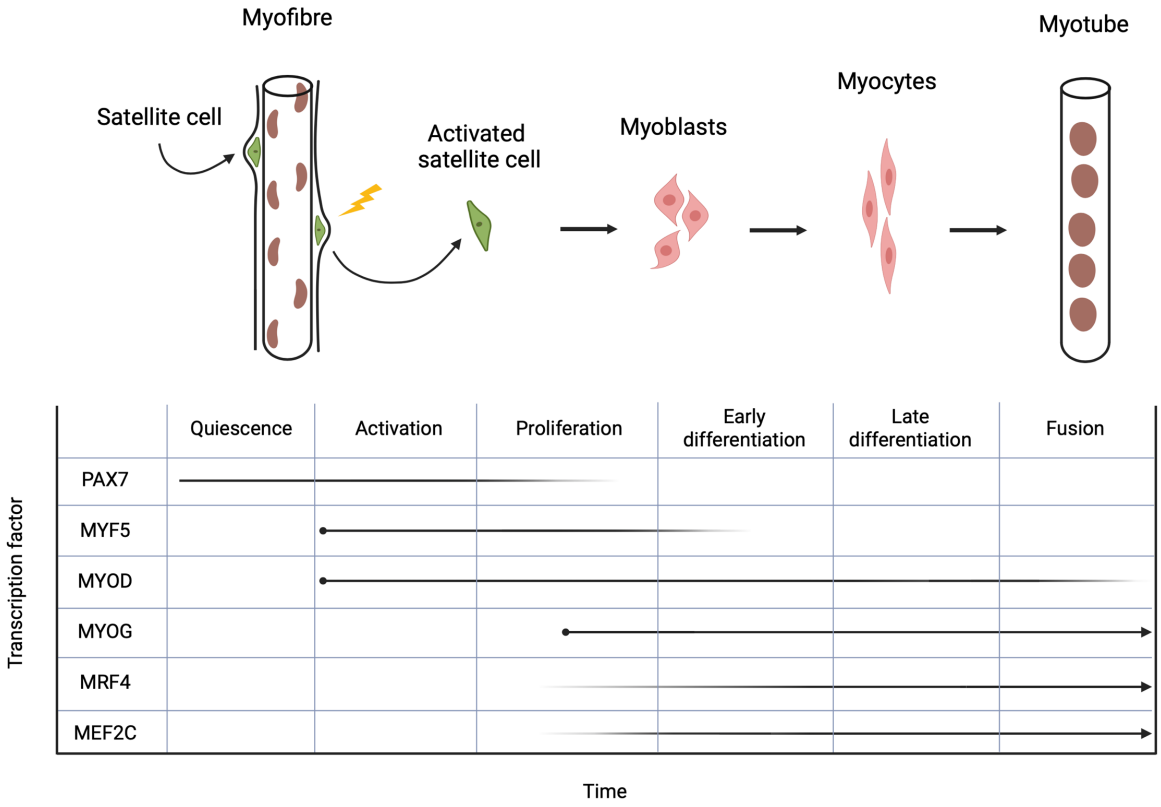

Figure 2. Transcription factors expressed at specific stages of the adult myogenesis. Skeletal muscle regeneration is a multistep process tightly regulated by a network of transcription factors. The expression of each transcription factor is stage specific and therefore precisely controlled. (Figure modified from Bentzinger et al., 2012)

1.1.3.1 The paired-box-containing transcription factors

The genes that encode the transcription factors of the PAX family are remarkably conserved and play crucial roles during embryonic tissue specification and organ development in many animal species (Vorobyov and Horst, 2006). In the context of myogenesis, PAX3 and PAX7 are of great importance. The formation of progenitor cells begins under the basal lamina of the myofibre during late embryonic muscle development. Ultimately, these cells gain a position which is characteristic for the satellite cells in adult muscle and are PAX3 and PAX7 positive. These not only regulate the entry of progenitor cells into the myogenic program but also control various aspects of their subsequent behaviour in regard to their fate during myogenesis (Relaix et al., 2005). Quiescent satellite cells in adult muscle are

uniformly strongly PAX7 positive, as it is essential for quiescence and self-renewal, thus for the maintenance of the satellite cell lineage (Figure 2) (Gnocchi et al., 2009; Kuang et al., 2008; Seale et al., 2000). It has been reported that mice deficient for *Pax7* show a complete absence of satellite cells. Furthermore, the cultivation of primary cells isolated from *Pax7*$^{-/-}$ mice did not give rise to satellite cell derived myoblasts. Upon muscle injury satellite cells exit the quiescent state and initially maintain PAX7 expression high, however, it decreases as proliferating myoblasts approach myogenic differentiation *in vivo* (Seale et al., 2000). It has also been shown that PAX7 blocks myogenic differentiation and progression in satellite cells by downregulating the expression of a myogenic regulatory factor MYOD, which results in an inhibited activation of another myogenic regulatory factor, Myogenin (MYOG) (Olguin and Olwin, 2004). Interestingly, when the C2C12 satellite cell derived cell line was studied *in vitro*, low levels of PAX7 were found in proliferating myoblasts similarly to what is observed *in vivo* (Seale et al., 2000).

1.1.3.2 Myogenic regulatory factors

The group of MRFs comprises four muscle specific proteins that belong to the basic-helix-loop-helix (bHLH) transcription factor family: MYF5, MYOD, Myogenin (MYOG) and MRF4. To describe their temporally and spatially tightly controlled expression patterns and to unravel their evident hierarchical relationships, was a crucial step towards understanding both the formation of the skeletal muscle lineage during embryonic development, and myogenic differentiation during postnatal myogenesis (Figure 2). During embryonic development MRFs together with PAX7 cooperatively establish the myogenic lineage by controlling the cell cycle arrest of the precursor cells (Hernández-Hernández et al., 2017). On the other hand, during postnatal myogenesis they control myogenic differentiation and thereby maintain the terminal skeletal muscle phenotype (Asfour et al., 2018).

Quiescent satellite cells in adult muscle along with high levels of PAX7 reportedly have low levels of MYF5 protein as well as non-translating mRNAs captured in messenger ribonucleoprotein (mRNP) granules allowing rapid translation. Thus,

quiescent satellite cells are transcriptionally primed by an MRF for a rapid start into myogenesis (Crist et al., 2012). After muscle injury when satellite cells become active, but before entering S-phase, they swiftly upregulate the expression of either MYF5 or MYOD or both simultaneously. After satellite cells entered the cell cycle and gave rise to proliferating myoblasts, most cells express only MYOD and a small proportion of them co-express both MYF5 and MYOD, which might be the result of a cross-activation between MRFs. Interestingly, later when committed myocytes differentiate and fuse together forming myotubes, RT-PCR analysis found that some of them do not express MYF5. Thus, satellite cells that never upregulated MYF5 expression despite activation could still proceed through proliferation, differentiation and fusion (Cooper et al., 1999; Cornelison and Wold, 1997; Cossu et al., 1966). Therefore, myoblasts that co-express MYF5 and MYOD would commit to myogenesis and differentiate, however myoblasts only expressing MYF5 without MYOD would continue to proliferate. *Myf5* mutant mice showed a delay in muscle regeneration, which interestingly became insignificant during the following steps of myogenesis. Furthermore, in satellite cell cultures derived from *Myf5* mutant mice a remarkable decrease in proliferation rate could be observed resulting in a delayed transition to myogenic differentiation (Ustanina et al., 2007). This suggests a role for MYF5 in transient myoblast amplification without being able to initiate myogenic differentiation.

MYOD, on the contrary, is necessary and sufficient to initiate myogenic differentiation. The expression of MYOD starts when upon muscle injury satellite cells become active. In non-muscle cells under physiological conditions MYOD expression is repressed. Astonishingly, MYOD overexpression in such cells activates the myogenic program, and suppressing other cell fates leads to the formation of differentiated muscle (Weintraub et al., 1991). Lack of MYOD delays the entry of satellite cells into cell cycle resulting in increased number of satellite cells, which increases the odds of satellite cell self-renewal rather than entering the myogenic program. In proliferating myoblasts MYOD deficiency is associated with proliferation defects and fewer cell divisions as compared to physiological conditions. This dysregulation leads to a decrease in myofibre number resulting in decreased muscle mass. Paradoxically, C2C12 myoblasts in culture lacking MYOD are hampered in their transition from proliferation to differentiation, they amplify

longer even in culture medium that is designed to promote differentiation. Thus, MYOD on one hand via regulating the expression of an inhibitory HLH protein ID3 represses the activity of other MRFs, thereby promoting undisturbed myoblast proliferation (Wyzykowski et al., 2002). On the other hand, MYOD activates genes involved in cell cycle progression and terminal differentiation. MYOD deficient mice after significant weight loss and gradual decay of the skeletal muscle tissue eventually die prematurely (Macharia et al., 2010; Megeney et al., 1996; Yablonka-Reuveni et al., 1999). At the molecular level, MYOD creates an open chromatin structure at muscle specific genes, which primes them for transcription driven by the next MRF in line, MYOG (Singh and Dilworth, 2013). This function of MYOD is pivotal for both undisturbed transition from proliferation to differentiation phase as well as for proper terminal differentiation.

Even though MYOG and MYOD act together to drive muscle differentiation, it is MYOG that at this point facilitates myogenesis via its strong antiproliferative activity (Liu et al. 2012). The transcriptional activity of MYOG initiates a gene expression program which shuts down the cell proliferation machinery, bringing amplifying myoblasts to cell cycle arrest and transitioning them into myocytes. As a permanent consequence of this fate decision, i.e. entering myogenic differentiation, there is no return from this committed state (Singh and Dilworth, 2013). MYOG is also expressed in newly fused myotubes and later in mature myofibres. The functions of other MRFs overlap to some extent with those of MYOG, however MYOG is indispensable. In MYOG knockout mice myogenesis virtually fails, resulting in only a few differentiated myofibres and the mice die perinatally (Hasty et al., 1993; Nabeshima et al., 1993). Thus, MYOG is essential until birth for survival. However, *Myog* deletion in mice after birth resulted in a 30% reduced body weight, but the animals retained normal skeletal muscle regeneration. Thus, in postnatal life MYOG is essential in maintaining body homeostasis (Knapp et al., 2006; Meadows et al., 2008).

The last MRF that acts downstream from MYOG is MRF4. This transcription factor has the highest transcript levels of all MRFs and in adult skeletal muscle is exclusively expressed in post-mitotic myocytes, myotubes and myofibres, thus during differentiation, fusion and final maturation (Gayraud-Morel et al., 2007;

Tajbakhsh et al., 1996). It is a myogenic determination factor during embryonic development since together with other MRFs, it participates in the establishment of the myogenic lineage by regulating progenitor cells (Weintraub et al., 1991). Loss of MRF4 in adult rat leads to muscle hypertrophy, increased protein synthesis and enhanced MEF2 (see below 1.1.3.3) target gene expression, which could be recapitulated by overexpressing MEF2 without affecting MRF4 (Moretti et al., 2016). This suggests a role for MRF4 in adult muscle in the maintenance of muscle homeostasis by repressing MEF2 activity resulting in the repression of genes responsible for hypertrophy.

1.1.3.3 The MADS-box containing transcription factor family

Four of the MADS-box (MCM1, Agamous, Deficiens, SRF) containing Myocyte enhancer factor 2 (MEF2) transcription factor family members can be found in vertebrates (MEF2A, B, C and D). MEF2 family members play pivotal roles in the differentiation, development and maintenance of diverse cell types such as neurons, hematopoetic cells and myofibres (Pon and Marra, 2015).

The MEF2 family member MEF2C starts to be expressed during late myoblast proliferation, but at this stage its activity is repressed (Potthoff and Olson 2007; Liu et al. 2014). Then during early differentiation, it becomes active, and its expression remains high during the following steps of myogenesis (Figure 2). By itself it does not possess myogenic activity, but in combination with MYOD and MYOG, it drives and amplifies the myogenic differentiation program acting as a transcription enhancer (Molkentin et al., 1995). It initiates cell cycle arrest and promotes terminal differentiation and fusion of committed myocytes (Badodi et al., 2015). It has been reported that in Drosophila *Mef2c* silencing had a severe effect on embryonic myogenesis, thus myoblasts lost their ability to fuse. The same effect was observed in adult myoblasts which lack *Mef2c*. In contrast, *Mef2c* silencing in mature myofibres in a post-fusion state had a mild effect on myogenesis, thus its role is taken over by other factors (Bryantsev et al., 2012). In line with that, muscle-specific silencing of *Mef2c* causes disorganized sarcomeres, disrupts myofibre integrity and

leads to perinatal death. It has been shown in Drosophila that MEF2C regulates the expression of genes encoding structural and sarcomere component proteins such as myomesin, myozenin, myotilin, myosin light and heavy chain, sarcoglycans and components of the troponin complex (Black and Olson, 1998; Potthoff and Olson, 2007). Furthermore, it regulates the expression of genes required for thick filament formation in zebrafish (Hinits and Hughes, 2007). MEF2C activity can be repressed by binding to its MEDS/MEF2 domains by the transcriptional co-repressors class II histone deacetylases (HDACs) 4 and 5 as well as by MITR (MEF2 interacting transcription repressor) resulting in the downregulation of myogenic target genes (Miska et al., 1999; Sparrow et al., 1999). HDAC-mediated repression of MEF2C is lifted by calcium-dependent signals, stimulating kinases that phosphorylate HDACs and MITR. Once phosphorylated, they are exported from nucleus, thus derepressing MEF2C target genes like MYOD and MYOG (Zhang et al., 2002). Additionally, MEF2C can be activated by MAP kinase signalling via direct phosphorylation by p38a which promotes the formation of a functional MYOD-MEF2C heterodimer (Brennan et al., 2021; Zhao et al., 1999). Furthermore, MEF2C is able to induce its own expression in a positive feedback loop (Wang et al., 2001). Our group has revealed a new transcriptional co-regulator, nTRIP6, which plays a role in the regulation of MEF2C transcriptional activity in myoblasts (see below 1.2.2).

1.1.4 Replenishment of the stem cell pool

Mature myofibres are in a post-mitotic state, thus postnatal muscle growth and regeneration depends on the pool of adult specific stem cells, the satellite cells. In order to maintain skeletal muscle homeostasis, these cells must self-renew. Two mechanisms have been described that contribute to the replenishment of the satellite cell pool: asymmetric cell division and reserve cell formation (Figure 3).

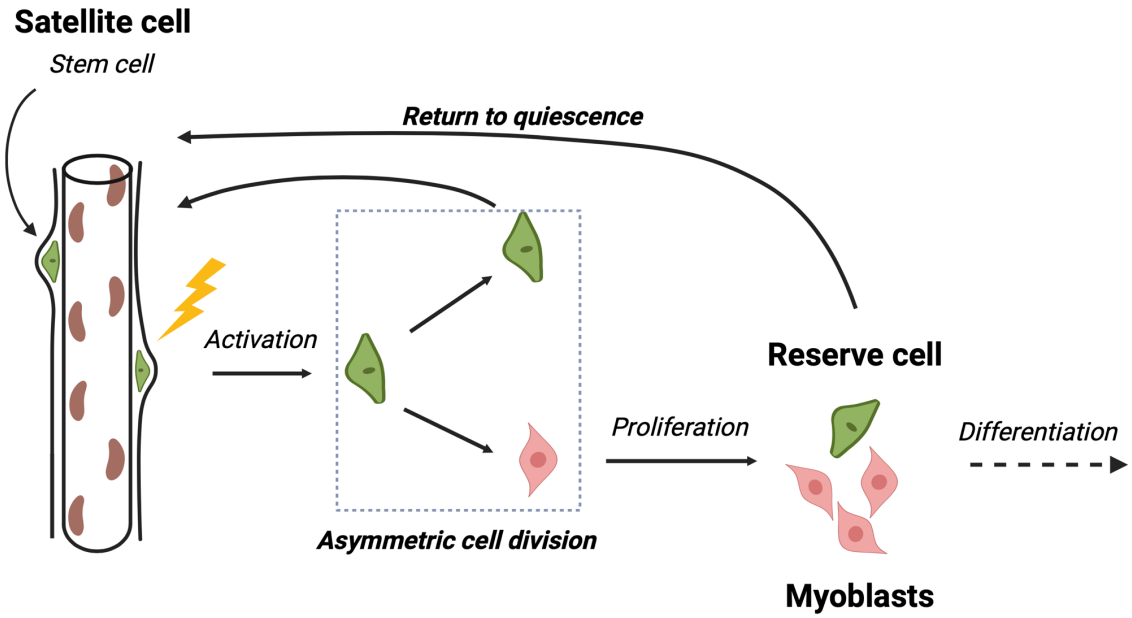

Figure 3. Replenishment of the adult stem cell pool. Upon muscle damage, satellite cells become active and undergo either a symmetric or an asymmetric cell division. Upon asymmetric cell division, one daughter cell remains a stem cell and returns to quiescence, the other one starts to proliferate as a myoblast and eventually enters differentiation. Proliferating myoblast can also return to quiescence as reserve cells thereby replenishing the satellite cell pool.

Under physiological conditions satellite cells are reversibly quiescent and in an undifferentiated cell cycle-arrested state. In nature, quiescence serves as a survival mechanism that sustains viability under unfavourable conditions. Thus, it stands to reason that it needs to be actively maintained in order to preserve the stem-function in satellite cells and to prevent their spontaneous differentiation (Rumman et al., 2015). Upon muscle injury satellite cells become active, stop being mitotically quiescent and enter the cell cycle. Their PAX7 expression, which in satellite cells is high, decreases as they start to proliferate as myoblasts. It has been described that PAX7 induces the expression of ID2 and ID3. These inhibitory HLH proteins interact with and inhibit the DNA binding of myogenic bHLH proteins, the MRFs, thereby preventing differentiation (Kumar et al., 2009). Thus, the decrease in PAX7 levels during myoblast proliferation participates in the promotion of myogenic differentiation. It has been shown that PAX7 also plays a role in satellite cell self-renewal (Kuang et al., 2008; Kuang et al., 2007). The fate choice whether a satellite cell self-renews or not is determined by two distinct types of mitotic events:

symmetric or asymmetric division. Sustaining the balance between these two events is crucial for stem cell maintenance and tissue homeostasis. In case of symmetric cell division, both daughter cells downregulate PAX7 as described above and induce MYOD and MYOG expression, which leads to their differentiation, thereby contributing to muscle regeneration. However, if a satellite cell divides asymmetrically (Figure 3), only one daughter cell proceeds to differentiation, the second one withdraws from the myogenic program, regains stem cell characteristics thereby replenishing the satellite cell pool (Conboy and Rando 2002; Kuang et al., 2007). Quiescent satellite cells can be identified as PAX7+ and MYOD- cells, activated satellite cells or proliferating myoblasts are PAX7+ and MYOD+, and differentiating cells are PAX7- and MYOD+ (Olguin et al., 2007; Zammit et al., 2004).

Making use of the *in vitro* differentiation of the C2C12 mouse myoblasts as a model of myogenesis, the formation of so-called reserve cells (Bouche et al., 2023; Laumonier et al., 2017; Yoshida et al., 1998) has been shown to contribute to the replenishment of the quiescent stem cell pool (Figure 3). As described above, upon muscle injury satellite cells become active and start to proliferate as myoblasts, during which they first upregulate the expression of MYOD and MYF5. Half of these cells then upregulate MYOG and undergo myogenic differentiation, fusion and eventually myofibre maturation. Surprisingly, the other half of the proliferating myoblasts do not engage with these events but rather escapes the differentiation program. These cells swiftly downregulate MYOD and MYF5 transcription factors, withdraw from the cell cycle, regain quiescence, and maintain an undifferentiated state. These cells can be isolated from a fully differentiated mature culture and put into growth medium. Interestingly, similarly to the original culture, they enter cell cycle and upregulate MYOD expression. They again give rise to proliferating myoblasts from which one half commits to differentiation, and the other half becomes MYOD negative and regains stemness. These cells that remained undifferentiated under differentiation promoting conditions were termed reserve cells (Yoshida et al., 1998).

In cell-based therapies for skeletal muscle regeneration satellite cell derived myoblasts are mostly used. However, clinical trials have revealed a reduced viability, migration and proliferation of the injected myoblasts (Mendell et al., 1995;

Skuk and Tremblay, 2015). Thus, the isolation and subsequent *in vitro* amplification of satellite cells reduces their overall regenerative potential. Human reserve cells on the other hand represent a promising alternative to satellite cells as they are very similar to human satellite cells and are easily expandable *in vitro*. In order to further investigate the regenerative potential of these cells, they were isolated from human primary myoblast cultures and transplanted into immunodeficient mice. Subsequent characterisation of these cells revealed that 80% of the human reserve cells were quiescent PAX7+ and MYOD- cells. Additionally, they demonstrated an improved viability and higher regenerative potential as they generated higher yields of PAX7+ quiescent cells as compared to satellite cell derived myoblasts (Laumonier et al., 2017). A recent study has shown that these *in vitro* generated reserve cells are a heterogenous population and can be divided into two groups based on their PAX7 expression. One reserve cell subpopulation had low levels of PAX7, thereby having more potential to commit towards myogenic differentiation. However, the other population with a high PAX7 expression profile was kept in a deeper quiescent state, less primed for differentiation displaying stronger stemness (Bouche et al., 2023). Thus, reserve cells, particularly those with high PAX7 expression, have the potential to replenish the quiescent stem cell pool *in vivo*, therefore they qualify as a potential therapeutic tool in muscle healing.

The mechanism determining these cell fates and thus regulating reserve cell formation is unknown and poses many questions. It remains unclear whether additional transcription factors are involved, or at which stage is the decision made, i.e. is it stochastically decided or pre-determined. It is described that reserve cells originate from proliferating myoblasts that remain undifferentiated, exit cell cycle and return to a quiescent state while rapidly losing MYOD expression, however the mechanism behind MYOD downregulation is unknown (Yoshida et al., 1998). A plausible hypothesis suggests that the initial step in reserve cell formation involves the shutdown of the activity of the myogenic transcription factors driving the differentiation program only in a subset of cells. This suppression suggests the involvement of transcriptional co-repressors. Notably, our group has recently shown that nTRIP6 (see below 1.2.2), the short isoform of a LIM domain protein, acts as a transcriptional co-repressor in myoblasts and prevents myoblast differentiation (Norizadeh Abbariki et al., 2021).

1.2 LIM domain proteins

The LIM domain-containing proteins were named after the three homeodomain proteins in which LIM domains were originally identified: Linl-1, Isl-1 and Mec-3 (Freyd et al., 1990). Proteins of this large and heterogeneous group can contain from one to five LIM domains, which are double zinc finger sequence motifs with conserved distribution of cysteine- and histidine-rich residues joined by a 2-amino acid spacer. Various zinc fingers which are present in diverse proteins such as transcription factors, kinases and adaptor proteins, mediate binding to DNA, RNA, lipids or other proteins (Gaullier et al., 1998; Mackay and Crossley, 1998). The LIM domain zinc fingers in particular mediate protein-protein interactions. This property of the LIM domain accounts for a wide range of functions in which LIM domain proteins are involved. Accordingly, it is present in at least 40 distinct proteins in eucaryotes. LIM domain proteins were initially classified based on their biological functions, then according to their LIM domains position and overall structural similarities (Dawid et al., 1995; Taira et al., 1995). Eventually, with the increasing number of LIM domain proteins, they were divided into four groups. The first group consists of LHX proteins and LIM-domain-only (LMO) proteins containing 2 LIM domains serving as transcription factors or cofactors and are localised in the nucleus. The second group comprises LMOs with two or more LIM domains, however these can be both localised in the nucleus and in the cytoplasm. Proteins with additional protein-protein interaction motifs, i.e. PDZ, LD (leucine-aspartate repeat) and ATD (actin-target domain) belong to the third and the fourth group, from which the fourth group additionally harbours a monooxygenase or a kinase catalytic motif. One subfamily of the third group, the ZYXIN subfamily, comprises proteins that have an N-terminal proline-rich part and three LIM domains in the C-terminal part such as ZYXIN, TRIP6 (thyroid receptor interacting protein 6), LPP (lipoma preferred partner) and Ajuba (Beckerle, 1997). They predominantly localise in the cytosol due to their leucine-rich nuclear export signals (NES) in their N-terminal part. In the cytosol, they primarily act as adaptor proteins and perform various functions such as the regulation of cell adhesion, cell migration, the assembly of the actin cytoskeleton and signal transduction. However, these LIM domain proteins have also been described in the nucleus where they regulate transcription (Zheng and

Zhao, 2007). They have therefore been proposed to shuttle from the cytosol to the nucleus (Wang and Gilmore 2003).

1.2.1 The LIM domain protein TRIP6

TRIP6 is a focal adhesion LIM domain protein which belongs to the ZYXIN subfamily. Its name, thyroid receptor interacting protein 6, derives from its identification in a two-hybrid screen as a protein interacting with the thyroid hormone receptor, however its function remained unknown (Lee et al., 1995). It is 476 amino acids long, contains three C-terminal LIM domains and is localised in the cytoplasm due to a functional nuclear export signal (NES) in its N-terminal pre-LIM region (Figure 4) (Wang and Gilmore 2001). TRIP6 is an adaptor protein which is enriched at sites of focal adhesion plaques and cell-cell contacts, where it interacts with the cytoskeleton, regulates cell adhesion and migration (Lai et al., 2007). Harbouring multiple transactivation domains, TRIP6 is also described to act as a transcriptional co-regulator (Wang and Gilmore, 2001). Although it is only expressed in the cytosol, it was hypothesised that TRIP6 is able to translocate into the nucleus (Lin and Lin, 2011), but no evidence has been found for that. On the contrary, our group demonstrated that TRIP6 does not perform these nuclear functions itself. Instead, these functions are carried out by a shorter isoform found exclusively in the nucleus, therefore termed nTRIP6 (Kassel et al. 2004).

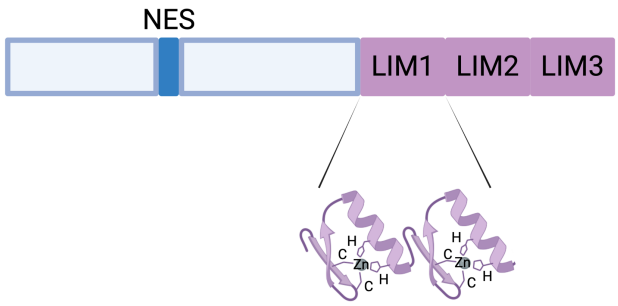

Figure 4. Schematic representation of the LIM domain protein TRIP6. TRIP6 harbours three C-terminal LIM domains each consisting of one pair of zinc finger sequence motifs, both coordinated by one zinc ion. In the N-terminal part of the protein a functional nuclear export signal (NES) is responsible for its cytosolic localisation.

1.2.2 The nuclear isoform nTRIP6

We have recently demonstrated that the two LIM domain protein isoforms, TRIP6 and nTRIP6, are generated from the same mRNA by alternative translation initiation (Fettig et al., 2023) (Figure 5). The *Trip6* mRNA contains two start codons (AUG) where the translation can potentially initiate. Translation initiation at the first one (AUG1) generates TRIP6, the complete form of the protein. This isoform harbours a functional NES in its C-terminal pre-LIM region, thereby it is localised in the cytosol. However, AUG1 is not in a perfect Kozak sequence, thus when the small ribosomal subunit (40S) binds the 5'-end of the mRNA and scans along to locate start codons for translation initiation, upon leaky scanning it can skip AUG1. In this case, it continues scanning until it reaches a second in frame initiation site (AUG2) located in the middle of the NES-encoding sequence. Thus, the initiation at this start codon produces the short isoform, nTRIP6. Due to its truncated, non-functional NES, it is exclusively localised in the nucleus (Fettig et al., 2023; Kemler et al., 2016).

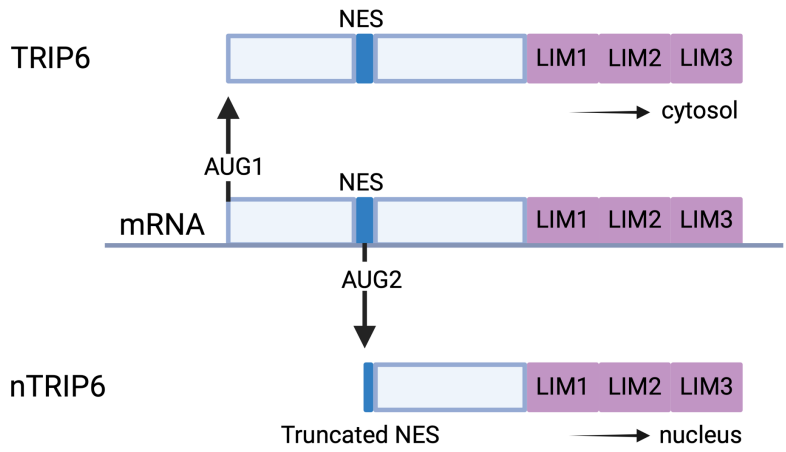

Figure 5. nTRIP6 is generated by alternative translation from the *Trip6* mRNA. TRIP6 and nTRIP6 are translated from the same mRNA. Translation initiation at AUG1 generates the longer isoform, TRIP6 which harbours a functional NES and is therefore localised in the cytosol. Translation initiation at AUG2 located within the NES-encoding sequence results in the shorter isoform nTRIP6 harbouring a truncated, non-functional NES, which is responsible for its nuclear localisation.

In the nucleus nTRIP6 interacts with and acts as a transcriptional co-activator for various transcription factors such as AP-1, NF-κB and the glucocorticoid receptor (Diefenbacher et al., 2008; Diefenbacher et al., 2010; Diefenbacher et al., 2014; Kassel et al., 2004). nTRIP6 exerts this transcriptional co-activator function as an adaptor serving as a platform for the recruitment of other regulatory proteins at the promoter of target genes. In particular, via its LIM domains it interacts with the Mediator complex protein THRAP3, and thereby mediates the recruitment of the Mediator complex to AP-1 target gene promoter regions (Diefenbacher et al., 2014).

1.3 nTRIP6 in postnatal myogenesis

In a yeast two hybrid screen an interaction has been reported between MEF2C and TRIP6 (Orchard et al., 2014). Given the role of MEF2C in late myocyte differentiation and fusion as well as in myotube maturation, this observation raised the possibility of a regulatory role for nTRIP6 in myogenesis. Indeed, our group has reported that in proliferating myoblasts nTRIP6 interacts with MEF2C, is recruited to the MEF2C binding regions of target genes, i.e. *Myom2* (Myomesin 2), *Mb* (Myoglobin), *Tnni2* (Troponin I2) and *Des* (Desmin). Surprisingly, nTRIP6 did not act as a co-activator for MEF2C but rather as a co-repressor, by mediating the recruitment of the class IIa histone deacetylase HDAC5 to the promoter of MEF2C target genes (Kemler et al., 2016). Furthermore, we have recently shown that nTRIP6 regulates the dynamics of myogenesis (Norizadeh Abbariki et al., 2021). Indeed, blocking nTRIP6 function accelerated the early differentiation of myoblasts, but delayed late myocyte differentiation and fusion. Thus, nTRIP6 appears to prevent premature differentiation of proliferating myoblasts. However, at the beginning of my work, the dynamics of nTRIP6 expression during myogenesis was not clear.

1.4 Hypothesis and aims

1.4.1 Regulation and function of nTRIP6 during postnatal myogenesis

Our group has shown that nTRIP6 prevents the premature differentiation of proliferating myoblasts (Norizadeh Abbariki et al., 2021), thereby it is involved in the regulation of early myogenesis. Thus, the aim of the first part of my work was to investigate the regulation of nTRIP6 expression over the course of myogenesis *in vitro*.

1.4.2 Role of nTRIP6 in the formation of reserve cells

The initial step in reserve cell formation involves the shutdown of the activity of the myogenic transcription factors driving the differentiation program in a subpopulation of proliferating myoblasts. Thus, via its anti-differentiation function nTRIP6 might be involved in this suppression (Norizadeh Abbariki et al., 2021). The aim of the second part of my work was therefore to investigate nTRIP6 involvement in reserve cell formation.

2. Materials and methods

2.1 Materials

2.1.1 Consumables

Unless stated otherwise, cell culture dishes, plates, and flasks were acquired from Greiner Bio-One GmbH (Frickenhausen, Germany).

2.1.2 Chemicals, solutions

Product	Company
ECL Prime Western-Blot-Detektionsreagenz	Cytiva Amersham
Lipofectamine 3000	Invitrogen
PeqGOLD TriFast™	Peqlab Biotechnologie
Protease Inhibitor Mix M (PIC)	SERVA Electrophoresis
Trypsin-EDTA	Gibco

Unless stated otherwise, all chemicals were acquired from Carl Roth GmbH&Co, (Karlsruhe, Germany), Gibco BRL (Eggenstein, Germany) or Sigma (Deisenhofen, Germany).

2.1.3 Hardware

Product	Company
ABI Prism Sequence Detection System 7000	Applied Biosystems
Cell incubator	Binder
Chemidoc Touch Imaging System	Bio-Rad Laboratories
Cytospin 4	Thermo Fisher Scientific
LSM 800	Zeiss
LSM 800 with Airyscan	Zeiss
Mini-PROTEAN Tetra Cell	Bio-Rad Laboratories

peqSTAR Thermocycler	Peqlab Biotechnologie
Sonicator	Diagenode Bioruptor
Trans-Blot Turbo Transfer System (midi)	Bio-Rad Laboratories

2.1.4 Antibodies

Primary antibodies

Antibody	**Isotype**	**Concentration**	**Company (order number)**
anti-glucocorticoid receptor (GR)	Mouse	1:1000 (WB)	Santa Cruz (sc-393232)
anti-HA	Rat	1:100 (IF)	Merck (11867423001)
anti-mCherry	Rabbit	1:1000 (IF)	ABCAM (Ab167453)
anti-MEF2C	Rabbit	1:1000 (WB)	Cell Signalling (5030T)
anti-MYH3	Mouse	1:100 (IF)	Developmental Studies Hybridoma Bank (F1.652-b)
anti-Myogenin (MYOG)	Rabbit	1:200 (WB)	Abcam (ab124800)
anti-PAX7	Mouse	1:1000 (WB)	Custom-made
anti-PAX7	Mouse	1:500 (IF)	Novus Biologicals (NBP2-32894)
anti-TNNI2	Goat	1:500 (WB)	Everest Biotech (EB12036)
anti-TRIP6	Rabbit	1:1000 (WB)	Custom-made
anti-β-actin	Mouse	1:25000 (WB)	Sigma Aldrich (A5441)

Secondary antibodies

Antibody	Isotype	Concentration	Company (order number)
Alexa Fluor 488-conjugated anti-mouse	Goat	1:1000	Invitrogen (A11001)
Alexa Fluor 546-conjugated anti-rat	Goat	1:1000	Thermo Fisher Scientific (A-11081)
Alexa Fluor 633-conjugated anti-rabbit	Goat	1:1000	Invitrogen (A21070)
α-goat- HRP	Rabbit	1:2000	Agilent Dako (P0448)
α-mouse- HRP	Goat	1:2000	Agilent Dako (P0260)
α-rabbit- HRP	Goat	1:2000	Agilent Dako (P0448)

2.1.5 Fluorescent dyes

Product	Company
4',6-diamidino-2-phenylindole, dihydrochloride (DAPI)	Thermo Fisher Scientific

2.1.6 Buffers

General buffers

DPBS	Phosphate buffered saline (Gibco)
RIPA	50 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% NaDoc, 0.1% SDS (supplemented with fresh PIC, PMSF)
TAE	40 mM Tris-acetate, 1 mM EDTA (pH 8,2 – 8,4)

Lowry protein assay

Lowry I	20 g/l Na_2CO_3, 4 g/l NaOH in ddH_2O
Lowry II	1% $CuSO_4$ in ddH_2O
Lowry III	2% Sodium potassium tartrate in ddH_2O
Lowry IV	Volume 100 Lowry I, volume 1 Lowry II, volume 1 Lowry III (freshly mixed)
Folin reagent	Diluted 1:1 in ddH_2O (freshly mixed)

SDS-PAGE and Western blot

Blocking buffer	5% Skimmed milk powder in TBS-T
Electrophoresis buffer	25 mM Tris-HCl, 192 mM glycine, 0.1% SDS
Separating gel	10% Acrylamide:bis-acrylamide, 1.5 M tris-HCl (pH 8.8), 0.1% SDS, 0.1% APS, 0.04% TEMED
Stacking gel	5% Acrylamide:bisacrylamide, 1 M tris-HCL (pH 6.8), 10% SDS, 10% APS, 0.1% TEMED
TBS-T buffer	20 mM Tris-HCl, 150 mM NaCl, pH 7.6, 0.05% TWEEN 20
Transfer buffer	200 ml Trans-Blot Turbo 5x, 200 ml 100% ethanol, 600 ml ddH_2O (Bio-Rad Laboratories)
2x Sample buffer	125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.01% bromophenol blue, 2% 2-mercaptoethanol

Immunofluorescence staining

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

2.1.7 Cell line and cell culture medium

C2C12	Mouse myoblast cell line (ATCC, LGC Standards GmbH, Wesel, Germany)
C2C12 culturing medium	Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal calf serum (FCS)
C2C12 differentiation medium	Dulbecco's Modified Eagle Medium (DMEM) with 2% horse serum

2.1.8 Bacterial strain and growth medium

E.coli	Chemically competent DH5α
Luria Broth (LB)	10 g/l yeast extract, 5 g/l NaCl, 5 g/l, tryptone (pH 7.5)

2.1.9 Plasmids

pcDNA3.1(+)	Empty vector, mammalian expression vector containing resistance for ampicillin and neomycin under the control of a CMV promoter (Invitrogen).
pcDNA3.1(+)-HA-Mm-nTRIP6-WT	An N-terminally HA-tagged nTRIP6 encoding sequence was cloned into an expression vector under the control of a CMV promoter (provided by Margarethe Litfin, IBCS-BIP, KIT).
pcDNA3.1(+)-mCherry-NLS	A functional nuclear localisation signal (NLS) sequence was fused to mCherry under the control of a CMV promoter (provided by Margarethe Litfin, IBCS-BIP, KIT).

2.1.10 Primers

Quantitative real time PCR primers (qRT-PCR)

Gene name	Forward primer (5' to 3')	Reverse primer (3' to 5')
Myog	GAGACATCCCCCTATTTCTACCA	GCTCAGTCCGCTCATAGCC
Rplp0	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
Tnni2	CATGGAGGTGAAGGTGCAGA	CTCTTGAACTTGCCCCTCAGG

Provided by ThermoFisher Scientific.

2.1.11 Peptide nucleic acids (PNA)

PNA	Sequence
AUG2	NH_2-GTCCAGATCAGCCAACAT-R_8-$CONH_2$
Mispaired control	NH_2-CTCCACATCACCCAAGAT-R_8-$CONH_2$

The AUG2 PNA targets the mouse *Trip6* mRNA sequence (NM_011639.3) from positions 481 to 498. The cell-penetrating moiety of the PNAs consists of an octoarginine peptide (R8). Due to the underlined mispaired bases in the sequence, the mispaired control is unable to bind. The PNAs were provided by the Vázquez laboratory (Marburg, Germany).

2.2 Methods

2.2.1 Cell culture methods

2.2.1.1 Cell culture conditions

C2C12 myoblast cells were cultured at 6% CO_2 and 95% humidity at 37°C. The cells were manipulated under a sterile hood and routinely checked for mycoplasma contamination. Non-sterile media, buffers and glassware were sterilized before work (120°C, 1.4 bar, 20 min).

2.2.1.2 Cell passaging

After aspirating the culturing medium, the cells were washed two times with PBS without Ca^{++} and Mg^{++} (PBS-/-). Then, the cells were treated with trypsin-EDTA and incubated for 3 minutes at 37°C. The trypsinization was stopped by adding culturing medium to the detached cells. After careful resuspension, the cells were centrifuged for 3 minutes at 1000 rpm. Then, the supernatant was aspirated, and the cells were resuspended in fresh culturing medium. To determine the cell number, a Neubauer chamber was used. Finally, cells were seeded at the required density:

Format	Cell number (per well)	Medium (per well)
15 cm dish	2.5 x 10^5	20 ml
6 well plate	5 x10^4	4 ml
24 well plate	1 x 10^4	1 ml

2.2.1.3 Differentiation of C2C12 myoblasts

The differentiation of C2C12 myoblasts occurred following a standardised protocol. Cells were seeded at low density (5 x 10^3/ cm^2) in culturing medium (DMEM supplemented with 10% FCS) at day -3, relative to the induction of the differentiation

at day 0. When the cells reached at least 80% confluence at day 0, the medium was changed to a differentiation medium (DMEM supplemented with 2% horse serum) which promoted myocyte differentiation and fusion. The medium was replaced every two days.

2.2.1.4 Separation of unfused cells from myotubes

C2C12 myoblasts were seeded according to a standardized protocol in a 24-well cell culturing dish (see 2.2.1.2) at day -3 relative to the induction of the differentiation (day 0). The cells were expanded in culturing medium (DMEM supplemented with 10% FCS). At day 0, the medium was changed to differentiation medium (DMEM supplemented with 2% horse serum) which promoted myocyte differentiation. In order to separate the fused myotubes from the unfused cells, a separation technique was adapted (Schoneich et al., 2014). First, a differential trypsinization was carried out using 0.025% trypsin-EDTA diluted in PBS-/-. This mild trypsinization step is ought to detach the myotubes first that tend to grow on the top of the unfused cells. The cell culture was incubated for 2 to 3 minutes at 37°C in this solution. To facilitate myotube detachment, the plate was swirled gently. The detached cells were added onto a strainer that was placed in a reaction tube. The myotubes were caught by the filter and remained on top, the unfused cells were collected in the reaction tube below. Then, a second trypsinisation step was carried out using the usual 0.25% trypsin-EDTA solution, upon which all cells were detached and subsequently strained. Finally, the strainer was washed using ice cold PBS-/- to remove mononuclear cell contamination. To collect the myotubes, the strainer was placed upside down on a reaction tube and washed again with PBS-/-.

2.2.1.5 Cell preparation for microscopy using Cytospin

To study the protein levels of unfused cells directly after separating them from the fused myotubes (see 2.2.1.4) during late differentiation and fusion, a cytocentrifuge was used (Cytospin 4, Thermo Fisher Scientific). First, the microscopic slide, filter

card and sample chamber were assembled, then the concentration of the cell suspension was set to $1x10^5$ cells/ ml in PBS-/-. $4x10^4$ cells were centrifuged onto a microscopic slide at 1250 rpm for 5 minutes, which then were directly fixed for immunofluorescent staining.

2.2.1.6 Collagen coating of coverslips

Sterilised coverslips (120°C, 1.4 bar, 20 min) were distributed in a 24-well cell culture dish under a sterile hood. 500 µl sterile filtered collagen solution (0.01% collagen and 0.2% acetic acid using collagen type I, Sigma-Aldrich) was added into each well and incubated for 30 minutes at room temperature. The solution was then aspirated, and the coverslips were left at room temperature to dry overnight. Cells were seeded and cultured on collagen coated coverslips in multiwell cell culture dishes for immunofluorescence experiments.

2.2.1.7 Cell transfection

Cells were seeded (see 2.2.1.2) on collagen coated glass coverslips (see 2.2.1.4) in a 24-well cell culture dish. After 24 hours incubation, these were transfected using Lipofectamine 3000. Appropriate amounts of DNA were diluted in serum-free medium and mixed with the P3000 reagent. The DNA mix and Lipofectamine 3000 were combined and incubated for 15 minutes at room temperature. Then, the transfection mixes were distributed in the wells. After 6 hours of incubation the medium was replaced by fresh culturing medium and the cells were incubated for additional 42 hours before fixation for immunofluorescence.

2.2.1.8 PNA treatment

C2C12 myoblasts were seeded in a 24-well cell culture dish either on collagen coated glass coverslips (see 2.2.1.4) or directly into the well. After 24 hours

incubation, the culturing medium was aspirated, and cell were treated with either the AUG2 PNA or the mispaired control (see 2.1.11) at a concentration of 10 µM in DMEM with reduced 2.5% FCS for 2 hours. Subsequently, the PNA solution was replaced by culturing medium (DMEM supplemented with 10% FCS), and the cells were expanded and differentiated if required (see 2.2.1.3).

2.2.2 Protein methods

2.2.2.1 Cell harvest for SDS-PAGE

Cells were washed two times with ice cold PBS-/- and lysed upon adding appropriate volumes of RIPA buffer (supplemented with a protease inhibitor mix). After scraping, the cell lysate was transferred into a 1.5 ml reaction tube and sonicated for 5 minutes at low intensity (20 seconds on, 20 seconds off). Then, the lyase was supplemented with 1 M DTT containing Laemmli sample buffer and boiled at 94°C for 5 minutes. After a short spin down, the sample was loaded on a sodium dodecyl-sulfate polyacrylamide gel for electrophoresis (SDS-PAGE).

2.2.2.2 Lowry protein assay

In order to measure the total protein concentration of a cell lysate, the Lowry protein assay was carried out. First, the Lowry IV solution was mixed and incubated for 5 minutes at room temperature (see 2.1.6 Lowry protein assay). 4 µl of the sonicated lysate (see 2.2.2.1) was mixed with 250 µl Lowry I and 500 µl Lowry IV solutions. Then, 50 µl Folin (1:1 in ddH_2O) was added and the mix was incubated for 30 minutes at room temperature in the dark. Two sets of BSA standards were mixed with known protein concentrations. The absorbances were measured spectrophotometrically at 595 nm wavelength. Based on the values of the standard curve, the total protein concentrations of the samples were calculated using the equation of a straight line.

2.2.2.3 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins by their size (Laemmli, 1970). 10 % polyacrylamide gels were cast according to Sambrook (Sambrook et al., 1989) and run in a Mini-PROTEAN Tetra Cell (Bio-Rad Laboratories) system for 1 hour at 100 V.

2.2.2.4 Western blot

After protein separation via SDS-PAGE, a semi-dry blotting was carried out in order to transfer the proteins onto a polyvinylidene difluoride (PVDF) membrane, which was activated using ethanol. The blotting sandwiches were soaked in Western blot transfer buffer (Bio-Rad Laboratories). For the transfer a Trans-Blot Turbo Transfer System (Bio-Rad Laboratories) was used at 1.3 mA/cm^2 for 7 minutes. The blotted membrane was first incubated in a blocking buffer (5% skimmed milk powder in TBS-T) for 1 hour, then with a primary antibody solution (diluted in blocking buffer) overnight at 4°C under constant shaking. Next day, the membrane was washed three times with TBS-T for 5 minutes and incubated with an HRP-conjugated secondary antibody (diluted in blocking buffer) for 1 hour at room temperature. The membrane was then washed three times in TBS-T. For signal detection the enhanced chemiluminescence Western blot kit (ECL Prime Western-Blot-Detektionsreagenz, Cytiva Amersham) and the ChemiDoc Touch Imaging System (Bio-Rad Laboratories) were used.

2.2.3 Immunofluorescence analysis

Immunostaining

C2C12 cells were seeded (see 2.2.1.2.) on collagen-coated coverslips (see 2.2.1.6) in a 24 well plate, then differentiated according to a standardized protocol (see 2.2.1.3). When they reached the required differentiation state, they first were

washed two times with ice cold PBS -/-, then fixed for 10 minutes at room temperature, washed again and permeabilized for 10 minutes (for buffers see 2.1.6). After washing, the cells were blocked in 5% BSA in PBS-/- for 1 hour at room temperature. The primary antibody was diluted in the blocking buffer, added to the cells, and incubated over night at 4°C. Next day, the cells were washed three times for 5 minutes with ice cold PBS -/-. Then, they were incubated in the secondary antibody diluted in blocking buffer for 1 hour at room temperature protected from light. Afterwards, they were washed and a nuclear counterstaining was carried out using DAPI diluted in ddH_2O for 15 minutes at room temperature. The coverslips were then washed again thoroughly and mounted (Aqua-Poly/Mount, Polysciences) on microscopic slides, these eventually dried at room temperature protected from light for 2 days.

Microscopy and analysis

Imaging of the differentiation experiment using the PNAs (see 2.1.11) and an anti-MYH3 primary antibody was performed using an LSM 800 confocal microscope (Zeiss). Images were acquired in tiling mode using a 10x/0.3 Plan-Neofluar objective resulting in 3 x 2 mm^2 images, which were analysed using Fiji. MYH3 positive mononuclear cells were studied by combining automated segmentation and manual counting. The percentage of nuclei within fused myotubes correspond to the fusion index. After analysis, linear brightness and contrast adjustments were made.

Imaging of the separated reserve cells (for separation see 2.2.1.4) incubated in an anti-PAX7 primary antibody, was carried out using an LSM 800 confocal microscope with Airyscan (Zeiss). Images were acquired in tiling mode using a 10x/0.3 Plan-Neofluar objective. The image analysis was carried out using Fiji. 200 individual nuclei were studied by combining automated segmentation and manual counting. Mean PAX7 signal intensities of the individual nuclei were measured. After analysis, linear brightness and contrast adjustments were made.

Myoblasts were transfected with either an HA-nTRIP6 or an mCherry-NLS expression plasmid and incubated in anti-HA and anti-PAX7, or anti-mCherry and anti-PAX7 primary antibody solutions. The imaging was carried out using an LSM 800 confocal microscope with Airyscan (Zeiss). Images were acquired in tiling mode using a 10x/0.3 Plan-Neofluar objective. The image analysis was carried out using Fiji. 255 individual nuclei were analysed by combining automated segmentation and manual counting. Mean PAX7 and HA, or PAX7 and mCherry signal intensities of the individual nuclei were measured. After analysis, linear brightness and contrast adjustments were made.

2.2.4 RNA isolation and quantitative real time PCR (qRT-PCR)

Over the course of a differentiation experiment (see 2.2.1.3) total RNA was extracted from PNA-treated cells (see 2.1.11). The culturing medium was aspirated, and the cells were harvested in PeqGOLD TriFast (Peqlab Biotechnologie). The lysates were mixed with chloroform and vortexed for 1 minute. To precipitate the RNA, the aqueous phase was transferred into a fresh reaction tube and mixed with isopropanol. Then, the reaction tube was centrifuged for 10 minutes at 12000 x g. The supernatant was removed, and the pellet was washed in 75% ethanol. After the pellet dried, it was resuspended in 20 µl ddH_2O. Samples were subjected to a DNase I treatment and incubated for 30 minutes at 37°C. The enzyme was inactivated by incubation at 65°C for 10 minutes, after adding EDTA. For the first strand synthesis a random primer mix was added and incubated with the samples for 10 minutes at 70°C. Then, the reverse transcription mix was prepared and incubated as follows:

5x MLRVT Buffer	4 µl
dNTP (10 mM)	2 µl
MLRVT	1 µl
H_2O	3 µl

10 min	25°C
60 min	42°C
10 min	70°C
store	4°C

The synthesized cDNA was diluted in RNase-free water and the following mRNAs were quantified (ABI Prism Sequence Detection System 7000, Applied Biosystems): *Myog* (Myogenin), *Tnni2* and *Rplp0* for normalization (P0 gene of the large ribosomal subunit). Samples were prepared and incubated as follows:

2x SYBR green	10 µl
Primer (10 pmol/µl)	2 µl
cDNA	4 µl
H_2O	4 µl

5 min	95°C
30 sec	95°C
30 sec	60°C
30 sec	72°C
35 cycles	
5 min	72°C
store	8°C

2.2.5 Statistical analysis

Statistical analyses were performed using R. Where indicated, significant differences were assessed by two-sided t-test analysis with values of $P<0.05$ sufficient to reject the null hypothesis. A Bonferroni correction was applied when multiple comparisons were performed. Where indicated, Pearson coefficient (r) and linear regression significance were calculated to investigate the correlation between two variables.

3. Results

3.1 nTRIP6 regulates myoblast differentiation *in vitro*

Using the *in vitro* differentiation of C2C12 mouse myoblasts as a model of myogenesis, we have previously reported that nTRIP6 regulates the dynamics of myogenic differentiation (Norizadeh Abbariki et al., 2021). I studied the time course of nTRIP6 expression during C2C12 cell proliferation and differentiation. In order to correlate the expression levels of nTRIP6 to the differentiation status of the cells, I also studied the expression of MYOG (also known as Myogenin) as an indicator of early myocytic differentiation (Edmondson and Olson, 1989; Wright et al., 1989) as well as that of TNNI2, a late differentiation marker (Lin et al., 1994). The expression of nTRIP6 was very low at the beginning of the proliferation phase (Figure 6A). It then increased to reach a maximum when the cells were nearly fully confluent before the medium was changed to differentiation medium (day 0). This time point coincided with the start of MYOG expression (Figure 6B). Thus, nTRIP6 levels increase to a maximum prior to the differentiation of the proliferating myoblasts into early differentiated myocytes. nTRIP6 levels then decreased at later time points (Figure 6A), which coincides with the start of TNNI2 expression (Figure 6C). Thus, nTRIP6 levels decrease in committed myocytes which typically start to fuse into myotubes at day 2 (see Figure 9 for fusion).

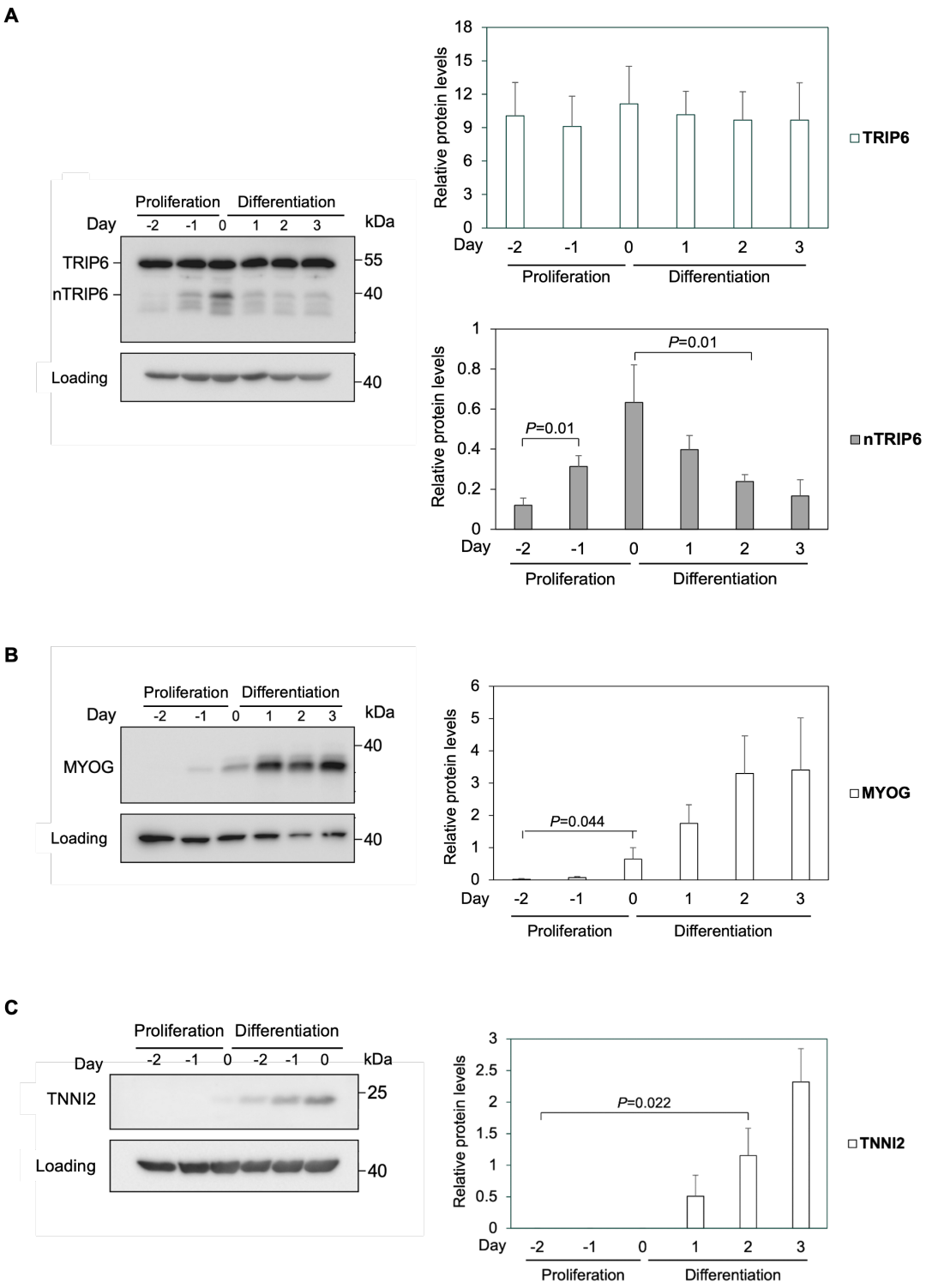

Figure 6. nTRIP6 expression transiently increases during myoblast differentiation. C2C12 myoblasts were subjected to a differentiation experiment and lysed at indicated days. Lysates were then analysed by Western blotting using an anti-TRIP6/nTRIP6 **(A)**, an anti-MYOG (Myogenin) **(B)** and an anti-TNNI2 antibody **(C)**. An anti-β-actin antibody was used as a loading control. Representative blots are shown (left panels). Protein levels relative to the β-actin loading control are presented as mean ± SD of three independent experiments (right panels).

Interestingly, the expression levels of the large cytosolic isoform TRIP6 remained constant over the course of the experiment (Figure 6A). We have recently shown that nTRIP6 is generated by translation initiation at an internal AUG (AUG2) in *Trip6* mRNA (Fettig et al., 2023). Therefore, considering that the expression of nTRIP6 is regulated during myogenesis while that of TRIP6 is not, a logical hypothesis is that nTRIP6 translation is transiently upregulated during the course of myoblast differentiation. To address this question, in collaboration with the Vázquez laboratory (Marburg, Germany) a peptide nucleic acid (PNA) conjugated to a cell-penetrating peptide (CPP) was designed to target AUG2 of *Trip6* mRNA in order to selectively inhibit nTRIP6 translation without affecting that of TRIP6. The AUG2 PNA and a mispaired version as a negative control were used in a C2C12 differentiation assay (Figure 7). TRIP6 expression levels were affected neither by the AUG2 PNA nor by the mispaired control PNA (Figure 7A,B). As expected, in the control treated cells nTRIP6 expression transiently increased during the course of the differentiation experiment, with a peak at the transition between proliferation and differentiation (Figure 7A,C). However, the AUG2 PNA abolished this transient increase. These results show that the transient increase in nTRIP6 expression is due to increased translation initiation at AUG2.

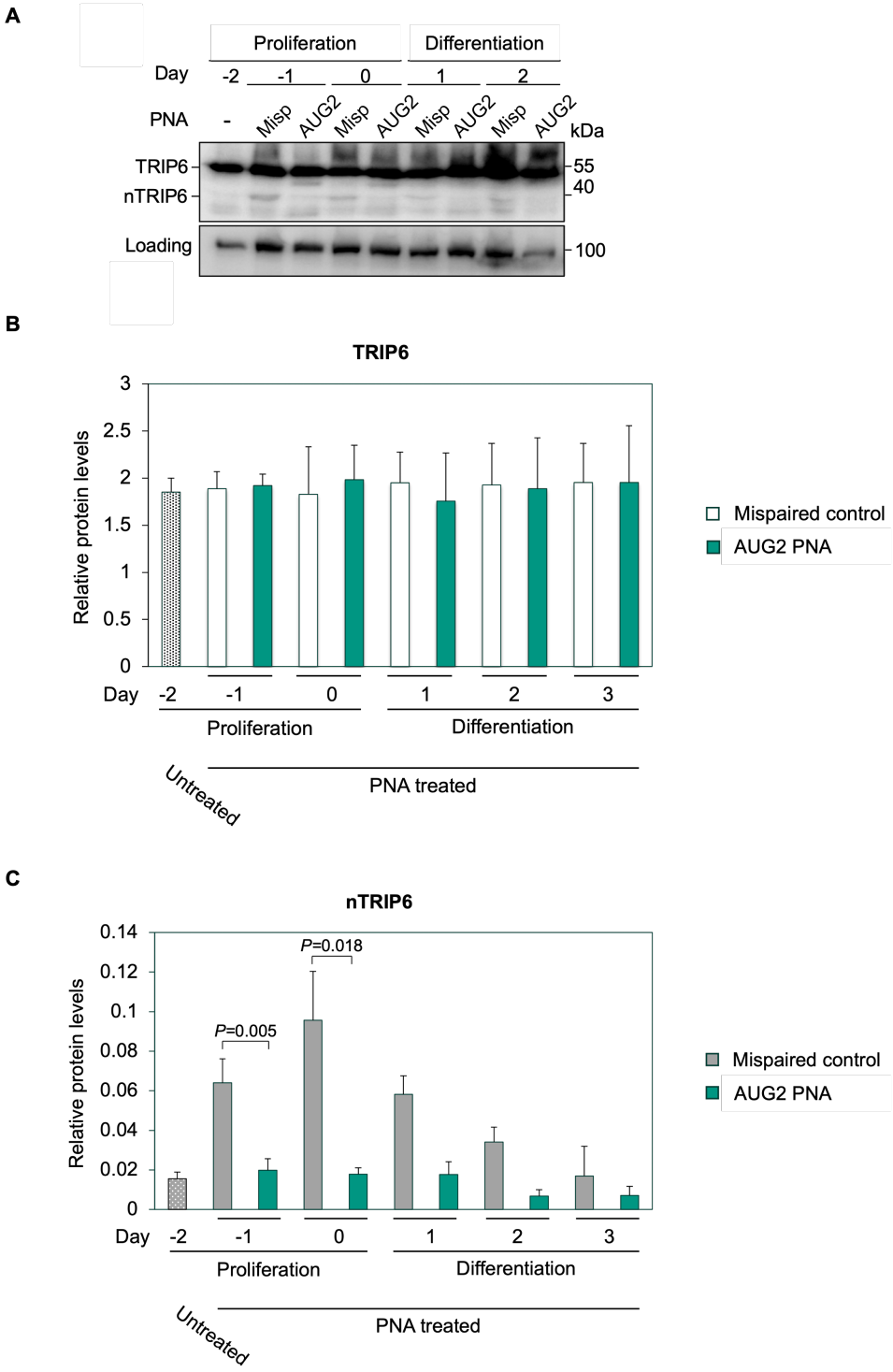

Figure 7. The transient increase in nTRIP6 expression occurs via increased translation initiation at AUG2. C2C12 myoblasts were subjected to a differentiation assay. At day -2 the cells were treated with either a cell penetrating peptide nucleic acid (PNA-CPP) targeting nTRIP6 translation initiation site (AUG2) or a mispaired negative control. Lysates were collected at the indicated days and analysed by Western blotting. **(A)** A representative blot is shown using an anti-TRIP6/nTRIP6 antibody and an anti-glucocorticoid receptor (GR) antibody as a loading control. **(B)** TRIP6 and **(C)** nTRIP6 protein levels relative to the GR loading control are presented as mean ± SD of three independent experiments.

We have previously shown that nTRIP6 inhibits premature myoblast differentiation, allowing proper myocyte differentiation and fusion at later stages (Norizadeh Abbariki et al., 2021). I used the AUG2 PNA to investigate whether this effect depends on the transient increase in nTRIP6 translation at early stages. To this end, I assessed the relative mRNA levels of the early differentiation marker *Myog* (Myogenin) and the late differentiation marker *Tnni2*. Myoblasts were treated at day -2 with either the target AUG2 PNA or the mispaired control, additionally an untreated control was included. In untreated control and in mispaired control PNA treated myoblasts the expression of the early myocytic differentiation marker *Myog* mRNA started as expected at the beginning of the differentiation phase, i.e. at day 0 (Figure 8A). However, in the AUG2 PNA treated myoblasts *Myog* mRNA expression was accelerated, starting already at day -1 and was significantly increased at day 0 as compared to control cells (Figure 8A). Conversely, the AUG2 PNA treated cells expressed significantly lower levels of *Tnni2* mRNA as compared to the mispaired control treated cells (Figure 8B), which indicates a delay in late differentiation. These results strongly suggest that selectively blocking nTRIP6 translation initiation at AUG2 accelerates early and delays late differentiation *in vitro*.

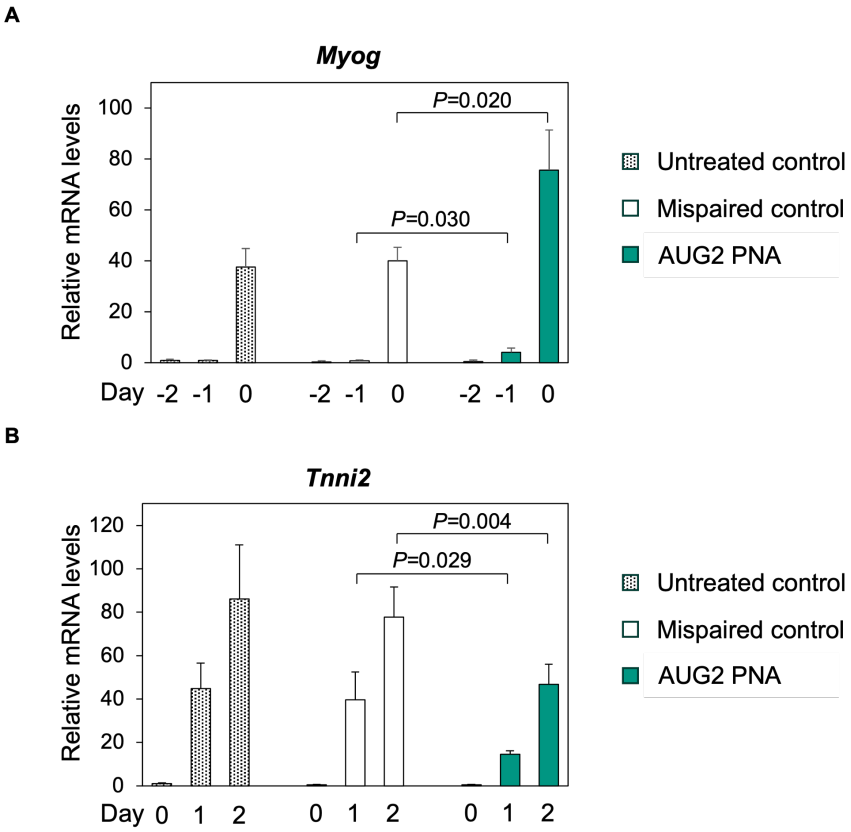

Figure 8. Blocking nTRIP6 translation initiation accelerates early and delays late differentiation. C2C12 myoblasts were subjected to a differentiation assay. At day -2 the cells were either untreated or treated with the AUG2 PNA or the mispaired control. Samples were harvested at the indicated days and mRNA levels were assessed by reverse transcription and real-time PCR. **(A)** *Myog* (Myogenin) and **(B)** *Tnni2* mRNA levels relative to the *Rplp0* gene are presented as mean ± SD of three independent experiments.

To further confirm the effect of blocking nTRIP6 translation initiation on late differentiation, I studied by immunofluorescence the expression of MYH3 (embryonic myosin heavy chain 3) which is the first muscle type myosin heavy chain expressed in differentiating myocytes (Schiaffino et al., 2015). As expected, at day 0 very few myoblasts expressed MYH3 (Figure 9A,B). In untreated control and in mispaired control PNA treated myoblasts the number of MYH3-positive cells strongly increased at day 1, when the differentiation of the myoblasts has already been set off. This increase was strongly inhibited in cells treated with the AUG2 PNA (Figure 9A,B). I then studied the effect of blocking nTRIP6 translation on myocyte fusion. In untreated control and in mispaired control PNA-CPP treated cells the fusion started at day 2 and strongly increased at day 3 (Figure 9C). Treatment with

the AUG2 PNA significantly inhibited fusion. Thus, blocking nTRIP6 translation initiation at AUG2 impairs late differentiation and fusion.

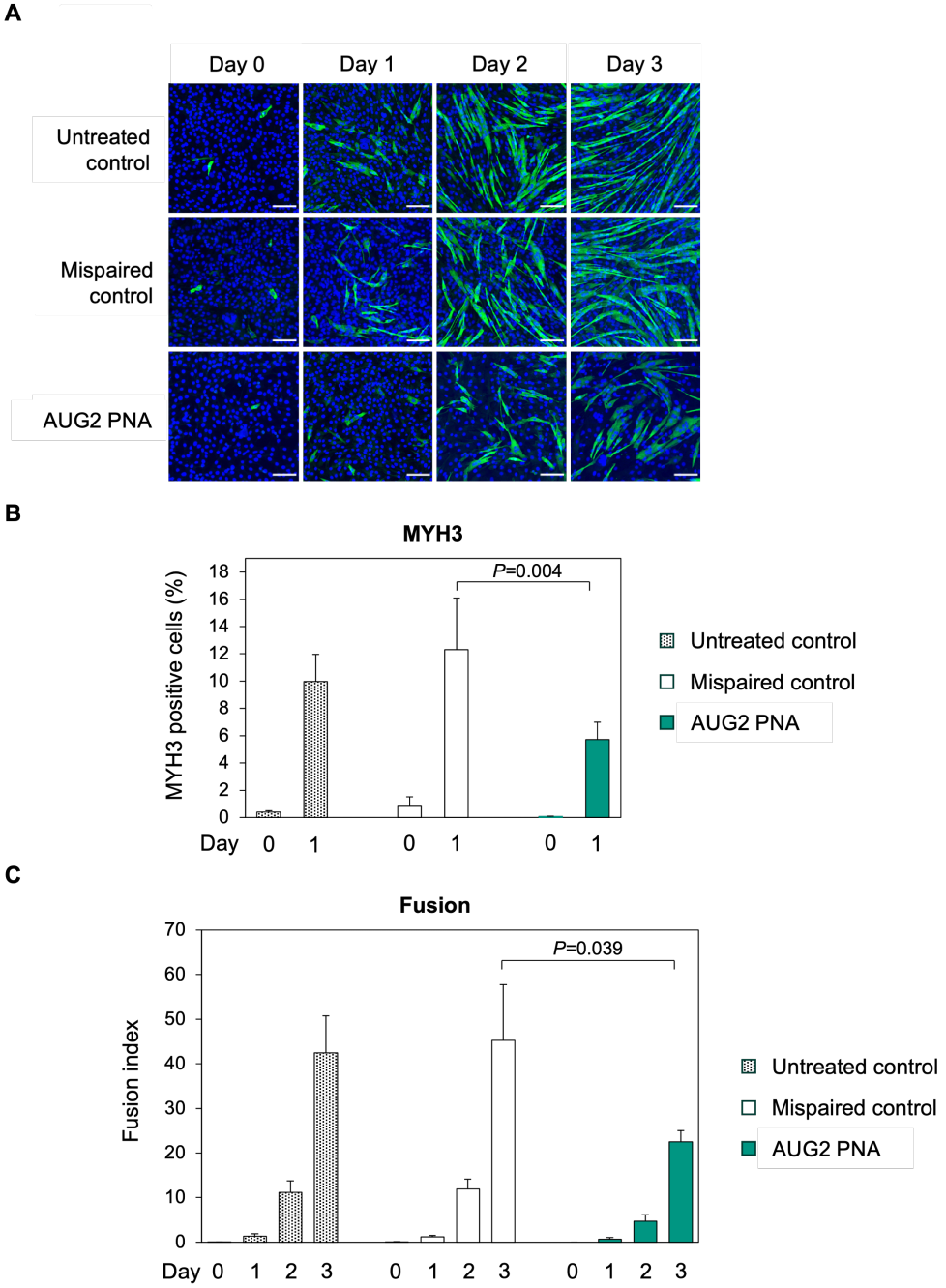

Figure 9. Blocking nTRIP6 translation initiation impairs late differentiation and fusion. C2C12 myoblasts were subjected to a differentiation assay. At day -2 the cells were either left untreated or treated with the AUG2 PNA or the mispaired control. The cells were fixed at the indicated days und subjected to immunofluorescence using an anti-MYH3 antibody and DAPI for nuclear counterstaining. **(A)** Representative images are shown (green: anti-MYH3; blue: DAPI; scale bar 200 µm). **(B)** The percentage of MYH3 expressing cells and **(C)** the percentage of nuclei within fused myotubes are presented as mean ± SD of three independent experiments.

Together, these results show that the transient increase in nTRIP6 translation at the end of the proliferation phase is responsible for the effects of nTRIP6 on the dynamics of myogenesis, i.e. the inhibition of premature myoblast differentiation to allow proper myocyte differentiation and fusion at later stages (Norizadeh Abbariki et al., 2021).

3.2 Function of nTRIP6 in the formation of reserve cells

Given the anti-differentiation function of nTRIP6 in the early stages of myogenesis, it can be hypothesised that nTRIP6 might play a role in the formation of reserve cells. The first question to address was how is nTRIP6 expressed in later stages of myogenesis. Thus, I have studied the time course of its expression throughout a prolonged differentiation experiment, until fusion is complete (day 11). As shown above, the expression levels of TRIP6 remained unaffected (Figure 10A,B) and those of nTRIP6 transiently increased to reach a maximum at the transition between the proliferation and the differentiation phase (Figure 10A,C). Then, during late differentiation nTRIP6 levels steadily decreased. However, even at late time points nTRIP6 expression did not decrease back to the starting levels (day -2). Instead, it was maintained even in the mature culture when the fusion was complete (Figure 10A,C). At intermediary time points the culture is heterogenous consisting of proliferating myoblasts, committed myocytes, already fused myotubes and possibly reserve cells. However, at day 11 fusion was complete, consisting of fused myotubes and reserve cells. Thus, cells at any of these differentiation stages could have contributed to the sustained levels of nTRIP6. Therefore, the next step was to identify the source of the maintained nTRIP6 expression.

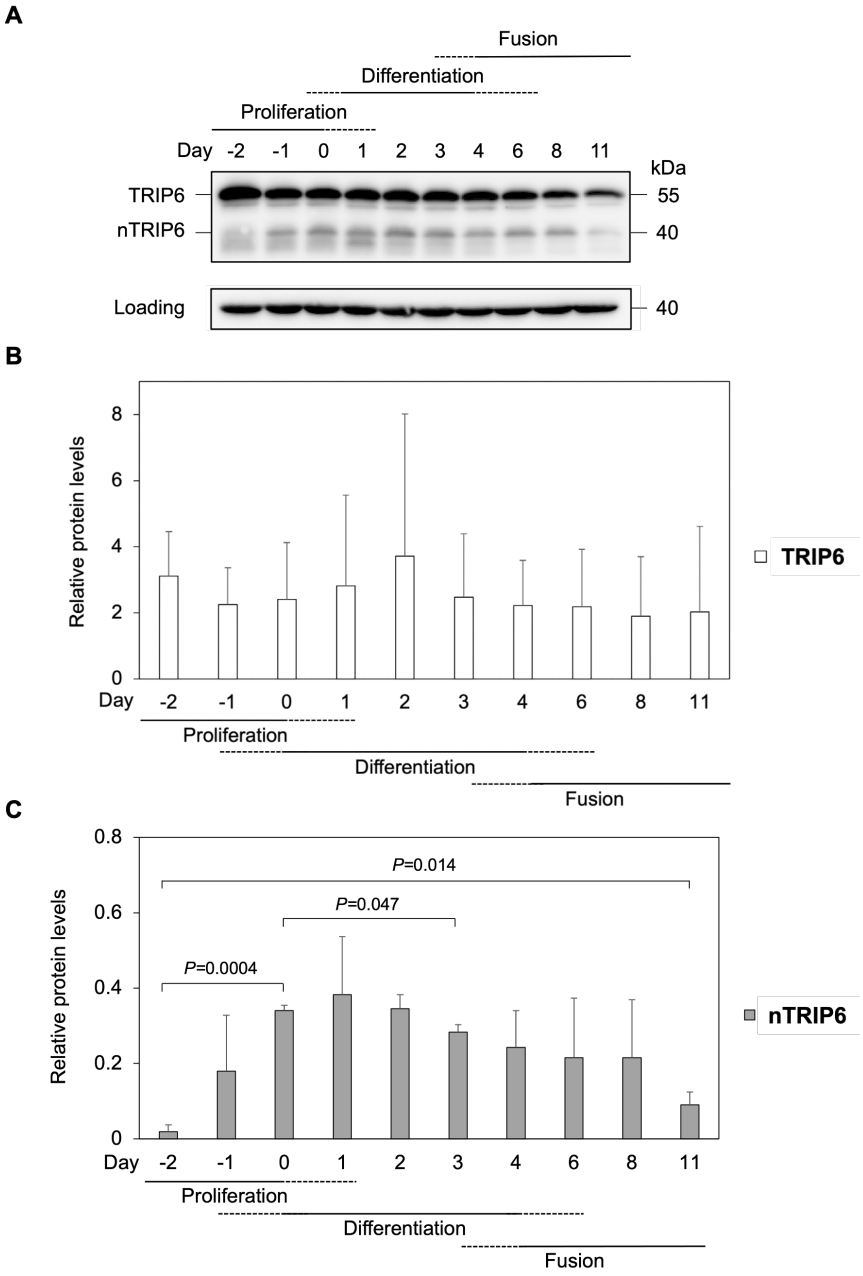

Figure 10. nTRIP6 expression is maintained during fusion. C2C12 myoblasts were subjected to a differentiation experiment and lysed at the indicated days. Lysates were then analysed by Western blotting using an anti-TRIP6/nTRIP6 antibody and an anti-β-actin antibody as a loading control. **(A)** Representative blots are shown. **(B)** TRIP6 and **(C)** nTRIP6 protein levels relative to the β-actin loading control are presented as mean ± SD of three independent experiments.

To address this question, I studied nTRIP6 expression in unfused cells exclusively. To this end a separation technique was adapted to remove myotubes from unfused cells (Schoneich et al., 2014). As expected, TRIP6 expression remained constant. (Figure 11A,B). Confirming my previous results, proliferating myoblasts at day -2

expressed very low amounts of nTRIP6 as opposed to day 0 (Figure 11A,C). Despite its decrease at later time points, its expression in unfused cells remained constantly higher than the starting levels (day -2). This suggests that nTRIP6 expression is maintained in a subpopulation of cells throughout differentiation and fusion. The sustained levels of nTRIP6 may inhibit their differentiation and thus contribute to the formation of reserve cells.

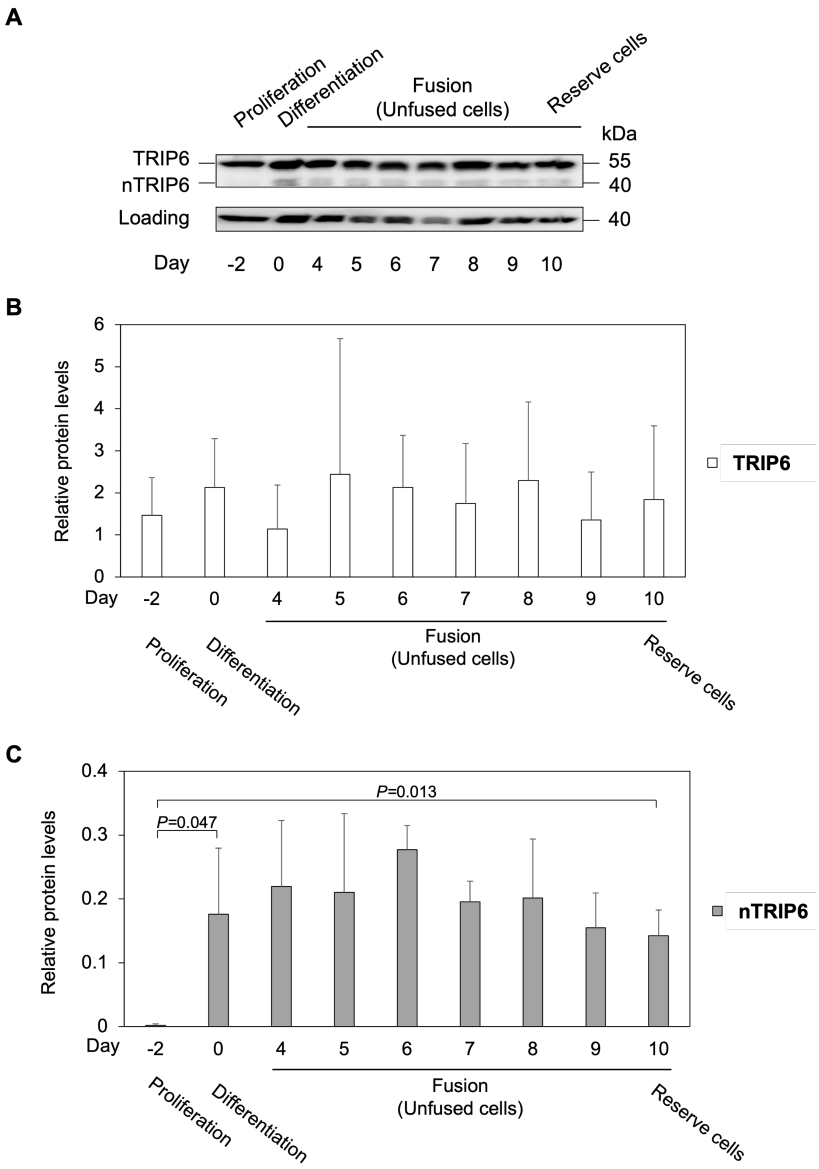

Figure 11. nTRIP6 expression is maintained in unfused cells during late differentiation and fusion. C2C12 myoblasts were subjected to a differentiation experiment and lysed at days -2 and 0. From day 4 until day 10 unfused cells were separated from myotubes. The lysates were analysed by Western blotting using an anti-TRIP6/nTRIP6 antibody and an anti-β-actin antibody as a loading control. **(A)** Representative blots are shown. **(B)** TRIP6 and **(C)** nTRIP6 protein levels are presented relative to β-actin loading control as means ± SD of three independent experiments.

Next, I investigated whether the dynamics of nTRIP6 expression in unfused cells is compatible with the appearance of reserve cells. Since reserve cells are quiescent, cycle arrested cells, I first studied the expression of the quiescence marker PAX7 in unfused cells (Gnocchi et al., 2009) (Figure 12A). In line with published data (Dey et al., 2011), there was a low PAX7 expression in proliferating myoblasts and early differentiated myocytes. This coincides with the transient increase in nTRIP6 expression peaking at day 0 (for nTRIP6 expression see Figure 11A,B). From day 6 its expression was maintained in unfused cells during fusion. Furthermore, unfused cells at day 10 express significantly higher levels of PAX7 as compared to myoblasts, which suggests that at day 10 the unfused cell population might predominantly consist of reserve cells. However, these Western blot results represent PAX7 expression in the entire population. Therefore, I performed single cell analysis of PAX7 expression by immunofluorescence (Figure 12B). As expected, proliferating myoblasts at day -2 expressed low levels of PAX7. Conversely, there was high PAX7 expression in all unfused cells at day 10, confirming that they are quiescent reserve cells.

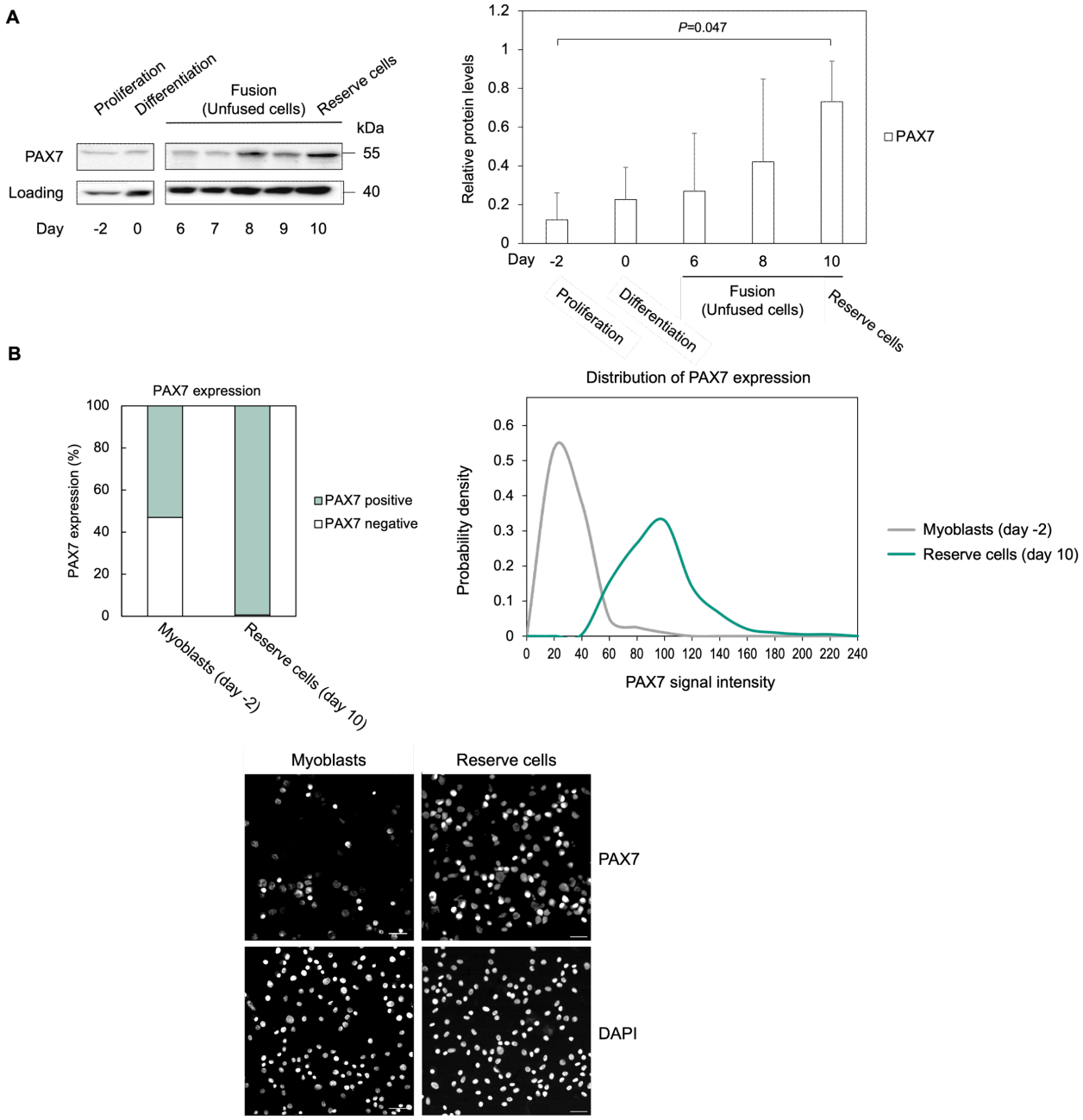

Figure 12. PAX7 is re-expressed and maintained in unfused cells during late differentiation and fusion. (A) C2C12 myoblasts were subjected to a differentiation experiment and lysed at days -2 and 0. From day 6 until day 10 unfused cells were separated and analysed by Western blotting using an anti-PAX7 antibody and an anti-β-actin antibody as a loading control. Representative blot is shown (left panel). PAX7 protein levels are presented relative to β-actin loading control as means ± SD of three independent experiments (right panel). **(B)** Myoblasts (day -2) and separated reserve cells (day 10) were subjected to immunofluorescence using an anti-PAX7 antibody and DAPI for nuclear counterstaining. The PAX7 signal intensity was determined in 200 individual nuclei. Results of a representative experiment out of three independent experiments are shown as the percentage of PAX7 positive and negative cells (upper left panel). The distribution of PAX7 signal intensity is presented as a probability density (upper right panel). Representative images are shown (lower panel) (scale bar: 50 µm).

I next investigated nTRIP6 expression in these quiescent reserve cells at day 10. For the characterisation of these cells, I first looked at the expression of the transcription factor MEF2C which is expressed in myoblast at the end of proliferation phase, in myocytes and in myotubes (Liu et al., 2014). The unfused cell fraction isolated from a mature culture at day 10, i.e. putative reserve cells, showed no expression of MEF2C. As expected, myotubes had high levels of MEF2C (Figure 13A,B). This indicates that the unfused cell fraction was not contaminated by myoblasts or myocytes. Furthermore, these unfused cells expressed PAX7, confirming that they are quiescent reserve cells. Conversely, there was no PAX7 expression in myotubes. As expected, reserve cells and myotubes showed no significant difference in TRIP6 levels. Strikingly, reserve cells showed high nTRIP6 expression as compared to myotubes. These results strengthen the hypothesis that nTRIP6 might play a role in the formation of reserve cells. The next question was whether nTRIP6 can indeed promote quiescence.

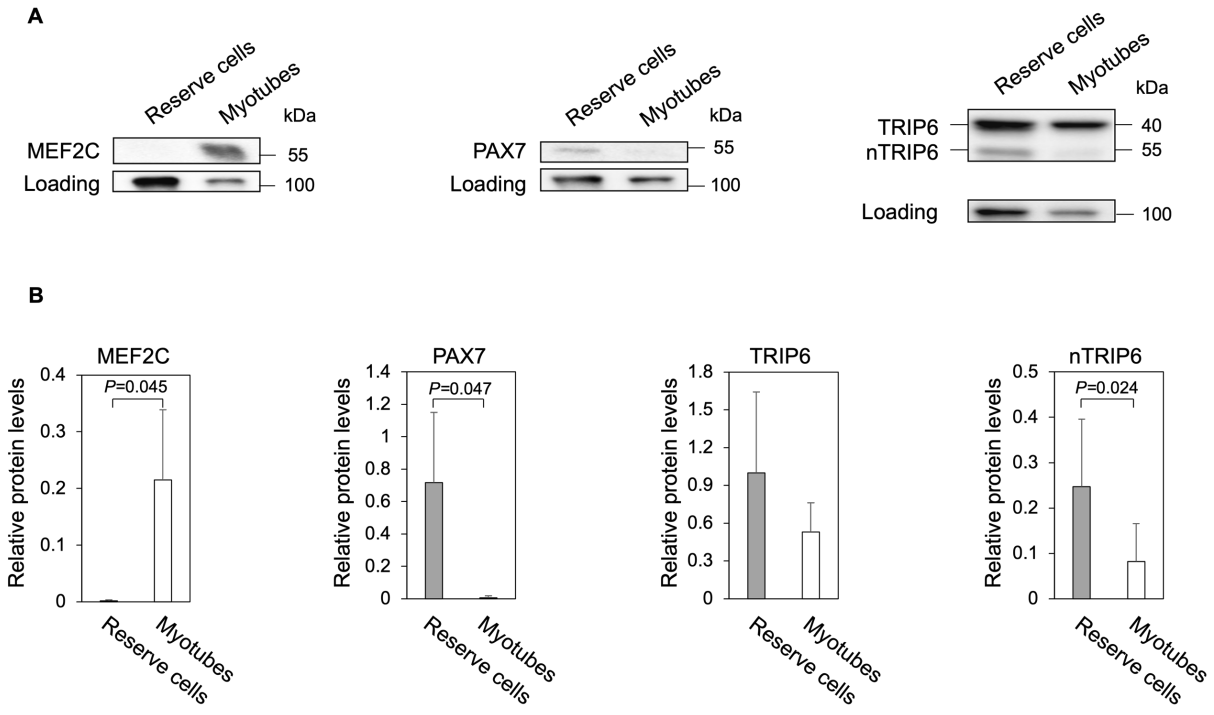

Figure 13. Reserve cells express high levels of nTRIP6. C2C12 myoblasts were subjected to a differentiation experiment. At day 10 reserve cells were separated from myotubes and analysed by Western blotting using anti-MEF2C, anti-PAX7 and anti-TRIP6/nTRIP6 antibodies. An anti-glucocorticoid receptor (GR) antibody was used as loading control. **(A)** Representative blots are shown. **(B)** MEF2C, PAX7, TRIP6 and nTRIP6 protein levels are presented relative to the loading control as mean ± SD of three independent experiments.

To address this question, I studied PAX7 expression, as a quiescence marker in myoblasts transfected with either an HA-tagged nTRIP6 expression plasmid or a nuclear-targeted mCherry plasmid of comparable size as control. After 48 hours cells overexpressing nTRIP6 had significantly higher PAX7 levels than the control cells (Figure 14A). Furthermore, at the single cell level, there was a strong correlation between overexpressed nTRIP6 levels and PAX7 expression (Figure 14B). Thus, nTRIP6 promotes quiescence in myoblasts.

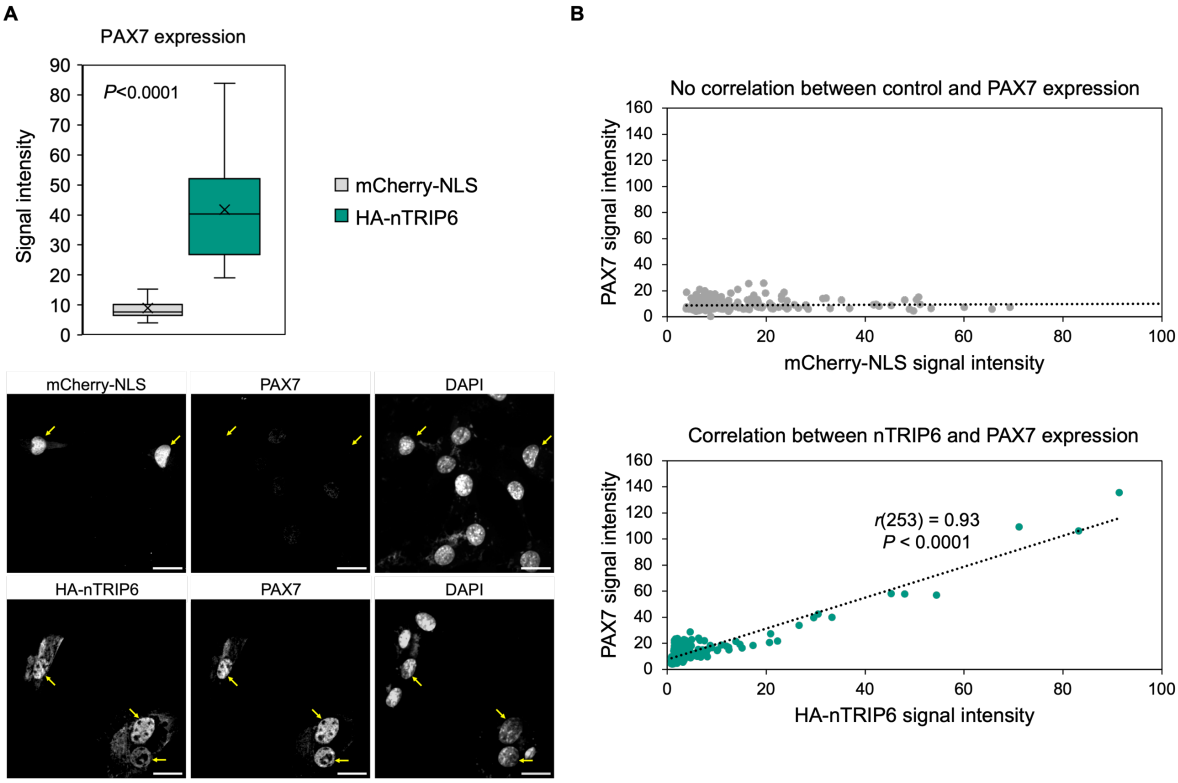

Figure 14. nTRIP6 increases PAX7 expression. C2C12 myoblasts were transfected with either an HA-tagged nTRIP6 expression plasmid (HA-nTRIP6) or a nuclear-targeted mCherry expression plasmid (mCherry-NLS) of similar size as a control. 48 hours after transfection cells were fixed and subjected to immunofluorescence using anti-HA and anti-mCherry antibodies and DAPI for nuclear counterstaining. Representative experiment is shown out of three independent experiments in which 255 nuclei were analysed per experiment **(A)** PAX7 signal intensities of the control plasmid transfected cells (mCherry-NLS) and of nTRIP6 overexpressing cells (HA-nTRIP6) are plotted (upper panel). Representative images are shown, in which arrows indicate the transfected cells (lower panel) (scale bar: 20 µm). **(B)** Correlation between nTRIP6 and PAX7 expressions in cells transfected with either the mCherry-NLS construct (upper panel) or the HA-nTRIP6 construct (lower panel) is plotted. Mean nuclear signal intensities are indicated as individual dots. A representative plot is shown out of three independent experiments. The Pearson coefficient (*r*) with degrees of freedom and linear regression significance (*P*) are presented.

4. Discussion

In this work, I have investigated the function of the transcriptional co-regulator nTRIP6 during postnatal myogenesis. First, I have shown that nTRIP6 expression transiently increases in proliferating myoblasts directly before they commit towards myogenic differentiation. Here, via its anti-differentiation function nTRIP6 prevents premature myoblast differentiation, which allows proper late myocyte differentiation and fusion. Furthermore, I have shown that the transient increase in nTRIP6 levels in proliferating myoblasts occurs via an increased translation initiation at an internal translation initiation site within the *Trip6* mRNA. Investigating the role of nTRIP6 in reserve cell formation, I have shown that a subpopulation of myoblasts maintains high nTRIP6 levels at late stages of myogenesis. When fusion is complete, the cells expressing high levels of nTRIP6 also express high levels of the quiescence marker PAX7, showing that they are reserve cells. Finally, I have shown that nTRIP6 promotes PAX7 expression in myoblasts. Thus, my results strongly suggest an involvement of nTRIP6 in the formation of reserve cells.

4.1 Regulation and function of nTRIP6 during postnatal myogenesis

In the first part of this thesis, I report that a transient increase in nTRIP6 translation occurs at the transition between myoblast proliferation and differentiation. This increase is required to prevent premature differentiation, and to allow proper differentiation and fusion at later stages.

My results show that nTRIP6 levels start to increase towards the end of the proliferation phase, but before MYOG (Myogenin) expression starts. Indeed, MYOG is an early differentiation marker which starts to be expressed at the myocyte stage (Edmondson and Olson, 1989; Wright et al., 1989), i.e. when myoblasts have stopped proliferating and engaged in differentiation. Furthermore, nTRIP6 expression strongly decreases when TNNI2, a late differentiation marker (Lin et al., 1994) starts to be expressed. Thus, nTRIP6 expression transiently increases during the transition from myoblast proliferation to differentiation.

Furthermore, I showed that the transient increase in nTRIP6 expression occurs via increased translation initiation at an internal AUG (AUG2). We have recently reported that nTRIP6 is generated via alternative translation which initiates at an internal AUG by a leaky scanning mechanism (Fettig et al., 2023). The first AUG in *Trip6* mRNA, at which the translation of the long cytosolic isoform TRIP6 initiates, is not in a perfect Kozak sequence. When the scanning ribosome skips this AUG, it initiates instead at a downstream AUG (AUG2), at which the translation of nTRIP6 initiates (Fettig et al., 2023). In *Trip6* mRNA AUG2 is located in the middle of a nuclear export signal (NES) encoding sequence (Wang and Gilmore, 2001). This NES is responsible for the cytosolic localisation of TRIP6 (Lin and Lin, 2011; Willier et al., 2011). Thus, nTRIP6 being translated from AUG2 harbours a truncated NES, which explains its nuclear localisation (Kassel et al., 2004; Kemler et al., 2016).

The observation that during the course of myogenesis TRIP6 expression is not regulated while that of nTRIP6 is transiently upregulated strongly suggested an increased translation selectively at AUG2. Indeed, treatment of myoblasts with the PNA to block initiation at AUG2 abolished the transient upregulation of nTRIP6. Thus, the increased nTRIP6 expression at the transition between myoblast proliferation and differentiation is the result of an increased translation. Furthermore, treatment with the PNA perfectly phenocopies the effect of blocking nTRIP6 function using a nuclear targeted inhibitory peptide (Norizadeh Abbariki et al., 2021), i.e. it accelerated early differentiation and delayed late differentiation and fusion. Therefore, the regulatory function of nTRIP6 in myogenesis is mediated by the selective upregulation of its translation.

What could be the mechanism behind the translational regulation of nTRIP6? We have recently reported that nTRIP6 translation is repressed by a short open reading frame (ORF) located immediately upstream of AUG2 (Fettig et al., 2023). This short ORF strongly resembles the upstream open reading frames (uORF) described in the 5' regulatory regions of many mRNAs, which repress the translation of the main ORF (Chen and Tarn, 2019; Zhang et al., 2019). Therefore, we have named this short repressing ORF in the *Trip6* coding sequence an iuORF, for internal upstream ORF. It is known that after uORF translation the mechanistic target of rapamycin complex 1 (mTORC1) can inhibit post-termination ribosome recycling resulting in an

increased re-initiation at the main ORFs downstream start codon (Gunišová et al., 2018; Schepetilnikov et al., 2013; Zidek et al., 2015). We have shown that at the transition between myoblast proliferation and differentiation, a transient increase in mTORC1 activity de-represses nTRIP6 translation without affecting TRIP6 levels (Fettig et al., 2023). Thus, it sems very likely that mTORC1 mediates the increase in nTRIP6 translation during early myogenesis by promoting translation initiation at AUG2 downstream of the repressing iuORF.

What could be the mechanism by which the transient increase in nTRIP6 translation prevents premature differentiation to allow proper late differentiation and fusion? I have observed that inhibiting the transient increase in nTRIP6 translation accelerates the expression of MYOG, an early differentiation marker (Edmondson and Olson, 1989; Wright et al., 1989), while it inhibits the expression of TNNI2 and MYH3, which are expressed later during differentiation (Lin et al., 1994; Schiaffino et al., 2015). Thus, the early increase in nTRIP6 translation prevents premature and promotes late differentiation. Our group has previously reported that nTRIP6 acts as a co-repressor for the transcription factor MEF2C in proliferating myoblasts (Kemler et al., 2016). It is known that MEF2C initiates cell cycle arrest and is therefore required for myocyte differentiation, and at later stages for fusion and myofibre maturation (Badodi et al., 2015). MEF2C expression is driven in particular by MYOD, a transcription factor which is expressed in proliferating myoblasts (Yablonka-Reuveni and Rivera, 1994). As a consequence, MEF2C is already expressed in proliferating myoblasts (Liu et al., 2014; Mokalled et al., 2012; Potthoff and Olson, 2007). However, some target genes of MEF2C encode sarcomeric and structural proteins (Black and Olson, 1998; Potthoff and Olson, 2007), which are only needed at later stages of myogenesis. Therefore, during early myogenesis MEF2C activity must be repressed. Indeed, our group has reported that nTRIP6 represses MEF2C transcriptional activity in myoblasts (Kemler et al., 2016). Therefore, the transient increase in nTRIP6 translation in early myogenesis is likely to repress MEF2C activity in proliferating myoblasts, and thus to prevent premature differentiation that would otherwise be driven by MEF2C. Indeed, *Myog* is a MEF2C target (Edmondson et al., 1992) and my results show that blocking nTRIP6 translation accelerates MYOG expression. MYOG induces cell cycle arrest in proliferating myoblasts and promotes their commitment towards differentiation

(Molkentin et al., 1995). Thus, the transient increase in nTRIP6 translation in proliferating myoblasts prevents premature differentiation.

My results also show that nTRIP6 is required for late myocyte differentiation and fusion. Indeed, blocking nTRIP6 translation initiation using the PNAs inhibited the expression of *Tnni2* mRNA and decreased MYH3 protein levels as well as delayed fusion. Since *Tnni2* is a MEF2C target gene (Nakayama et al., 1996), one would have expected an increased expression. However, when *Tnni2* and MYH3 expression start to increase, nTRIP6 expression has already decreased back to low levels. Therefore, a direct regulatory effect of nTRIP6 on their expression is unlikely. Furthermore, MEF2C activity is required for the expression of target genes which are needed at later stages of myogenesis, such as sarcomeric and contractile proteins (Black and Olson, 1998; Potthoff and Olson, 2007). At these stages, nTRIP6 levels have strongly decreased. Thus, it seems that the impaired late differentiation and fusion are rather an indirect consequence of blocking nTRIP6 function at earlier stages, i.e. the prevention of premature differentiation.

In conclusion, my results together strongly suggest that the early, transient increase in nTRIP6 translation is required to inhibit premature differentiation, which in turn is required for a proper myocyte differentiation and fusion at later stages.

4.2 Role of nTRIP6 in the formation of reserve cells

The second part of my work strongly suggests a role for nTRIP6 in reserve cell formation. Here I first report that nTRIP6 expression is maintained at relatively high levels in reserve cells. Indeed, in a prolonged differentiation experiment until fusion is complete, I found that after the early transient increase in nTRIP6 expression, nTRIP6 levels steadily decreased but remained elevated as compared to the starting levels. More specifically, nTRIP6 was expressed at high levels in unfused cells at late time points when fusion was complete. These cells also expressed high levels of the quiescence marker PAX7 (Gnocchi et al., 2009), confirming that they are reserve cells. Thus, reserve cells express high levels of nTRIP6.

The kinetics of nTRIP6 expression in the unfused cell population during late differentiation and fusion suggests that nTRIP6 expression is maintained at a high level in a subpopulation of cells, rather than being re-expressed at late stages. Given the anti-differentiation effect of high levels of nTRIP6 in the early stages of myogenesis, it is tempting to speculate that the subpopulation of unfused cells which maintains high levels of nTRIP6 escapes differentiation and fusion and enter a quiescent state to give rise to the reserve cell population. My observation that overexpression of nTRIP6 in proliferating myoblasts promotes an increase in the expression of PAX7 further supports this hypothesis.

Reserve cell formation via return to quiescence is a poorly understood mechanism. It is known that a subpopulation of already proliferating myoblasts escape myogenic differentiation, exit cell cycle and return to quiescence (Bouche et al., 2023; Laumonier et al., 2017; Yoshida et al., 1998). Given that via its anti-differentiation effect nTRIP6 prevents premature differentiation during early myogenesis (Norizadeh Abbariki et al., 2021), it can be hypothesised that the same mechanism is involved in preventing the differentiation and fusion of those myoblasts that are prone to become reserve cells. Specifically, nTRIP6 represses the transcriptional activity of MEF2C (Kemler et al., 2016), a transcription factor which by itself does not possess myogenic activity but in combination with other MRFs drives and amplifies differentiation (Molkentin et al., 1995). The first step towards myogenic differentiation would be cell cycle exit, which is induced by the MEF2C target MYOG. Therefore, it stands to reason that through its co-repressor function for MEF2C, nTRIP6 might downregulate MYOG expression and thereby prevent the differentiation of a subpopulation of myoblasts that maintains a high level of nTRIP6 expression during late differentiation and fusion.

It is known that differentiating cells are PAX7 negative, however it is highly expressed in cell cycle arrested cells (Gnocchi et al., 2009). Therefore, nTRIP6 positive myoblasts that re-expressed and maintained high PAX7 levels during late differentiation, entered a mitotically quiescent state and remained unfused. When myotube fusion was complete, these cells became reserve cells co-expressing high levels of nTRIP6 and PAX7. Additionally, I have shown that the overexpression of nTRIP6 leads to high PAX7 levels in myoblasts, indicating that nTRIP6 by itself can

induce a quiescent state in myoblasts. What could be the mechanism by which nTRIP6 promotes the return to quiescence? The simplest hypothesis is that via its transcriptional co-regulator function, nTRIP6 directly stimulates *Pax7* gene transcription. This remains to be investigated. However, another likely hypothesis is that nTRIP6 co-repressor function contributes to the promotion of quiescence. Along with the regulation of early myoblast differentiation via MYOG (Edmondson et al., 1992; Molkentin et al., 1995; Ridgeway et al., 2000), MEF2C is also involved in late myocyte differentiation, fusion and myotube maturation via other MRFs such as MYOD and MRF4 (Black et al., 1995; Molkentin et al., 1995; Moretti et al., 2016). MYOD is also known as the master regulator of myogenesis. It starts to be expressed in satellite cells directly after muscle injury (Bentzinger et al., 2012). Interestingly, MYOD has been shown to play a role in satellite cell activation via participating in the reduction of the condensation of the transcriptionally inactive heterochromatin (Boonsanay et al., 2016). Conversely, PAX7 has been reported to help create and maintain a condensed heterochromatin (Günther et al., 2013). Thus, MYOD and PAX7 have antagonistic functions in satellite cells. It has been widely studied that PAX7 depletion results in the complete absence of satellite cells, therefore PAX7 seems to be essential for quiescence (Gnocchi et al., 2009; Kuang et al., 2008; Seale et al., 2000). Additionally, a general reciprocal inhibition has been described between PAX7 and all MRFs (Olguin et al., 2007). Upon activation, PAX7 positive quiescent satellite cells upregulate MYOD expression, and the PAX7/MYOD ratio of the cell can determine its fate. A low PAX7/MYOD ratio induces MYOG expression and leads to terminal differentiation. Conversely, high PAX7/MYOD ratios lead to the downregulation of MYOD and may lead to satellite cell self-renewal maintaining the quiescent state. Interestingly, there is a cooperation between MYOD and MEF2C, in that MEF2C is required for MYOD activity (Molkentin et al., 1995) and both transcription factors reciprocally upregulate their expression (Wang et al., 2001). Thus, it might be hypothesised that, if in reserve cells nTRIP6 mediated repression of MEF2C affects MYOD expression and/or activity, it might contribute to the return to quiescence. Furthermore, since I report that nTRIP6 increases the expression of PAX7, it is tempting to speculate that nTRIP6 promotes reserve cell quiescence by increasing the PAX7/MYOD ratio. Thus, nTRIP6 might abolish the MYOD-mediated maintenance of open euchromatin as well as the proliferation promoting effect of MYOD in those myoblasts which

maintain high nTRIP6 and PAX7 levels. This putative function of nTRIP6 might put myoblasts in an inactive state where both proliferation and differentiation are blocked, which favours their transition into a mitotically quiescent state. This cell cycle arrest could promote PAX7 upregulation and chromatin condensation resulting in the formation of reserve cells. Additionally, PAX7 has also been described to block MRFs via inducing the expression of the inhibitory ID3 protein (Kumar et al., 2009). Thus, nTRIP6-mediated increase in PAX7 expression in future reserve cells might contribute to the repression of the myogenic program by itself. Taken together, via its co-repressor function for MEF2C, nTRIP6 might block proliferation and differentiation in a subpopulation of myoblasts during late differentiation and fusion thereby promoting cell cycle exit, PAX7 upregulation, chromatin condensation and eventually the return to quiescence. Thus, nTRIP6 might play a role in reserve cell formation.

Beside nTRIP6 co-repressor function for MEF2C, might there be another mechanism by which it promotes the return to quiescence? Our group has shown that nTRIP6 is not only a co-repressor of MEF2C, but also a co-activator for several transcription factors such as AP-1, NF-κB and the glucocorticoid receptor (Diefenbacher et al., 2008; Diefenbacher et al., 2010; Diefenbacher et al., 2014; Kassel et al., 2004). AP-1 is involved in the regulation of diverse cellular processes, i.e. growth, proliferation, differentiation and apoptosis. Interestingly, AP-1 also has tissue specific functions. Numerous MYOD target genes in skeletal muscle were found to possess AP-1 binding sites suggesting that similarly to MYOD, AP-1 might play a role in the promotion of the myogenic program. However, the function of AP-1 largely depends on the subunit composition of this dimeric transcription factor, which allows the execution of diverse biological functions (Andreucci et al., 2002). Paradoxically, the AP-1 subunit Fra-2 has been found exclusively expressed in PAX7 positive quiescent cells, such as satellite cells and reserve cells, and Fra-2 represses the myogenic program (Alli et al., 2013). Thus, a potential role has been proposed for Fra-2 in the maintenance of the undifferentiated state of reserve cells and satellite cells. This way AP-1 might be able to simultaneously execute an inhibitory function on the myogenic program and thereby to maintain quiescence in reserve cells and satellite cells, and at the same time to promote the progression through myogenesis. Interestingly, nTRIP6 interacts with Fra-2 and strongly

increases the transcriptional activity of Fra-2-containg AP-1 dimers (Diefenbacher et al., 2008). Thus, nTRIP6 might contribute to the quiescence of reserve cells also through its co-activator function for Fra-2-containing AP-1.

4.3 Conclusion

My work has shed new light onto the function of nTRIP6 in postnatal myogenesis. At early stages, via a transient increase in its translation at an internal AUG, nTRIP6 prevents premature myoblast differentiation. This early anti-differentiation effect is required for an undisturbed subsequent myocyte differentiation and fusion (Fettig et al., 2023; Norizadeh Abbariki et al., 2021). Thus, nTRIP6 regulates the dynamics of early postnatal myogenesis.

At later stages, the high expression levels of nTRIP6 in reserve cells together with the observation that nTRIP6 increases PAX7 expression strongly suggest that nTRIP6 contributes to the return to quiescence of reserve cells. Such a function might contribute to the replenishment of the skeletal muscle stem cell pool, and thus to the maintenance of the high regenerative capacity of the skeletal muscle.

In conclusion, I propose that nTRIP6, through its anti-differentiation function, has a dual role in regulating postnatal myogenesis: an early role in timing the onset of differentiation, and a late role for the replenishment of the quiescent cell pool.

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6. Publications

Fettig, R.*, **Gonda, Z.***, Walter, N.*, Sallmann, P., Thanisch, C., Winter, M., Bauer, S., Zhang, L., Litfin, M., Etard, C., Armant, O., Vázquez, O., and Kassel, O. (2023) *Short internal open reading frames regulate the translation of N-terminally truncated proteoforms.* bioRvix, doi:10.1101/2023.11.09.566418. (* equal contribution)

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