Non-Canonical Functions of SMAD3 and SMAD2 During Myogenic Differentiation and Fusion

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AUTHORIZATION

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ABSTRACT

The transcription factors SMAD2 and SMAD3 are the effectors of classical transforming growth factor beta (TGFβ) signalling. This signalling cascade is involved in many cellular processes including proliferation and differentiation and is known to be a potent inhibitor of myogenic differentiation through SMAD3. We have previously shown that retinoic acid (RA) can upregulate SMAD3 in models of adipogenesis and mesenchymal stem cells and that SMAD3 can interact with the bZIP transcription factor C/EBPβ to disrupt its DNA binding. Forced expression of C/EBPβ inhibits myogenic differentiation but the mechanism has not been fully elucidated. Herein we show that RA increases *Smad3* expression in myoblasts and that RA treatment antagonizes TGFβ-mediated inhibition of myogenic differentiation. TGFβ treatment increased C/EBPβ expression which was reversed by RA treatment. Further, RA was able to disrupt C/EBPβ occupancy of the *Pax7* and *Smad2* promoters in myoblasts. Loss of C/EBPβ in primary myoblasts using a conditional knockout model partially protected these cells from the anti-myogenic effects of TGFβ treatment.

The TGFβ effector protein SMAD2 is expressed in myoblasts but its specific function in myogenesis has not been determined, as *Smad2* knockout models are embryonic lethal. Thus, we created a novel *Smad2* conditional knockout model where *Smad2* is excised in PAX7-expressing muscle satellite cells. Herein we demonstrate a role for SMAD2 specifically in myogenic fusion. We describe a regeneration defect after acute injury and decreased fiber cross-sectional area at P21 (post-natal day 21) in $Smad2^{cKO}$ muscle, without affecting the numbers of PAX7-positive cells. Further, we reveal a mechanism whereby SMAD2 regulates KLF4 expression and mediates the KLF4-induced increased of the fusion gene *Npnt*. This work describes the pro-myogenic

actions of RA-induced SMAD3 and the novel function of SMAD2 in terminal myogenic differentiation and fusion. This work also discusses future directions, implications and new insights into non-canonical SMAD actions.

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LIST OF ABBREVIATIONS

ALK5: Activin-like kinase 5

bFGH: Basic fibroblast growth factor

bHLH: Basic helix-loop-helix

BMP: Bone morphogenic protein

bZIP: Basic leucine zipper

CBP: CREB-binding protein

C/EBPα: CCAAT-enhancer binding protein alpha

C/EBPβ: CCAAT Enhancer Binding Protein Beta

cAMP: Cyclic adenosine monophosphate

CDK: Cyclin-dependent kinase

CDK15: Cyclin dependent kinase 15

ChIP: Chromatin Immunoprecipitation

cKO: Conditional knockout

CTX: Cardiotoxin

DBD: DNA binding domain

DM: Differentiation medium

DMEM: Dulbecco's Modified Eagle Medium

EGF: Epithelial growth factor

FGF: Fibroblast growth factor

GM: Growth medium

HGF: Hepatocyte growth factor

IBMX: Isobutylmethylxanthine

IL-4: Interleukin 4

IL-6: Interleukin 6

i.p.: intraperitoneal

KLF4: Kruppel-like factor 4

LAP: Liver activating protein

MAD: Mothers against decapentaplegic

MH1: Mad homology domain 1

MH2: Mad homology domain 2

MRF4: Muscle regulatory factor 4

MRFs: Muscle regulatory factors

MYF5: Myogenic factor 5

MyHC: Myosin heavy chain

MYOD: Myogenic differentiation 1

NES: Nuclear export signal

NF-IL6: Nuclear factor interleukin 6

NLS: Nuclear localization signal

Npnt: Nephronectin

PAI-1: Plasminogen Activator Inhibitor type 1

PAX7: Paired box 7

PBS: Phosphate buffered saline

PBS-T: Phosphate buffered saline with Tween

P/CAF : p300/CBP-associated factor

qPCR: Quantitative Polymerase Chain Reaction

RA: Retinoic acid

RAR: Retinoic acid receptor

RARE: Retinoid acid response element

RXR: Retinoid X receptor

SARA: Smad anchor for receptor activation

SGF: Sarcoma growth factor

TA: Tibialis anterior

TGFβ: Transforming growth factor beta

TMEM8C: Transmembrane protein 8c (myomaker)

TNF-α: Tumour necrosis factor alpha

α-SMA: Alpha smooth muscle actin

XSA: Cross-sectional area

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CHAPTER ONE: INTRODUCTION

1.1 General Introduction

Skeletal muscle is the most abundant tissue in the human body, accounting for approximately 40% of total body weight (Frontera and Ochala 2015). Skeletal muscle is made up of differentiated muscle cells called myocytes, which arise from myoblasts, that fuse together to form multinucleated myofibers required for locomotion and support. This tissue has the ability to maintain and restore itself throughout life, for example after exercise or injury, due to resident adult stem cells called satellite cells. The molecular mechanisms governing myogenesis and regeneration are complex, with multiple signalling pathways and transcription factors contributing to this process. For the purpose of this thesis, the involvement of the Transforming Growth Factor β (TGFβ) pathway, mainly the effector proteins SMAD2 and SMAD3, retinoic acid (RA) signalling and CCAAT/Enhancer binding protein β (C/EBPβ) activity in the regulation of myogenesis will be in focus.

1.2 Skeletal myogenesis and the myogenic regulatory factors

Skeletal muscle development in the vertebrate embryo begins in the somites, a structure derived from the mesoderm. The somites further divide to form the sclerotome and the dermomyotome, the latter giving rise to the dorsal dermis and skeletal muscle. Cells derived from the myotome, termed muscle progenitors, migrate, proliferate and differentiate into multinucleated myofibers to form muscle of the limbs, trunk and tongue, a process known as myogenesis (reviewed in Buckingham et al., 2003). Mature muscle fibers are arranged into bundles of striated myofibers, containing thousands of myofibrils, and are enveloped by a basal lamina. Myofibrils are organized into repeating units called sarcomeres, comprised of the most abundant muscle contraction proteins actin and myosin, among other cytoskeletal proteins (reviewed in Frontera & Ochala, 2015). In the adult, skeletal muscle homeostasis is maintained

by the presence of resident adult muscle stem cells termed satellite cells, located between the sarcolemma and basal lamina of individual muscle fibers, which have the ability to both differentiate to repair injured muscle and to self-renew (Mauro 1961; Zammit 2002).

Adult skeletal myogenesis is a well-organized process governed by the induction and expression of transcription factors known as the myogenic regulatory factors (MRFs). The MRFs, including MYF5, MYOD, myogenin (MYOG) and MRF4, are part of the basic helix-loop-helix (bHLH) family of transcription factors, which bind E-boxes found in many myogenic promoters, and are known for their ability to convert non-myogenic cells to the myogenic lineage by upregulating muscle specific genes (Wright, Sassoon, and Lin 1989; Choi et al. 1990; Miner and Wold 1990; Braun et al. 1989, 1990; Davis, Weintraub, and Lassar 1987; Tapscott et al. 1989; Weintraub et al. 1989; Rhodes and Konieczny 1989). The knockout phenotypes for the MRFs were investigated in mice and revealed a sequential cascade of events controlling myogenic differentiation, placing MYF5 and MYOD as important myogenic commitment factors while myogenin and MRF4 are induced later in differentiation. Surprisingly, despite MYOD being a master regulator of skeletal muscle, *Myod*$^{-/-}$ animals have normal skeletal muscle suggesting that another factor can compensate for its loss *in vivo* (Rudnicki et al. 1992). Indeed, prolonged *Myf5* expression compensates for loss of *Myod* to ensure normal expression levels of myogenin and MRF4, though these mice have delayed regeneration after acute injury (Rudnicki et al. 1992; Megeney et al. 1996). *Myf5*$^{-/-}$ animals die shortly after birth of respiratory failure due to a malformed ribcage, but similar to *Myod*$^{-/-}$ animals, they have relatively normal skeletal muscle with unchanged expression of MYOD, myogenin and MRF4 (Braun et al. 1992). To understand the compensatory mechanisms involved in muscle development, MYF5 and MYOD double homozygous null mutants were characterized (Rudnicki et al. 1993). Compound *Myf5*$^{-/-}$

:*Myod*$^{-/-}$ mutants died soon after birth with a complete lack of muscle fibers and myoblasts. Additionally, these animals failed to upregulate myogenin and MRF4 expression, placing MYF5 and MYOD as important factors for the determination of myogenic cells in development. However, the phenotype of the *Myf5*$^{-/-}$:*Myod*$^{-/-}$ double knockout mutant was revisited as it was determined that disruptions in the *Myf5* locus also compromised MRF4 transcription due to their genetic proximity and shared *cis* regulatory elements (Kassar-Duchossoy et al. 2004). Indeed characterization of a new *Myf5*$^{-/-}$:*Myod*$^{-/-}$ mutant where MRF4 expression was not affected, showed these mutants formed skeletal muscle during embryogenesis and myogenic progenitor cells could differentiate in culture, suggesting that MRF4 acts as a determination factor (Kassar-Duchossoy et al. 2004).

Myogenin$^{-/-}$ mice die perinatally from respiratory failure owing to a severe reduction in all skeletal muscle, a non-functional diaphragm and a small ribcage (Hasty et al. 1993; Nabeshima et al. 1993). The skeletal muscles of *myogenin*$^{-/-}$ animals show major defects, demonstrated by an abundance of mononucleated cells and the rare presence of myofibers. Furthermore, while MYOD levels were normal, these animals failed to induce the expression of MRF4 and differentiation markers myosin heavy chain (MyHC) and actin, suggesting these myoblasts are committed to the myogenic lineage but fail to differentiate and form myofibers (Hasty et al. 1993; Nabeshima et al. 1993).

Mrf4 was the last of the myogenic regulatory factors to be inactivated in mice. The expression of this MRF was found to be transient; expressed early in embryogenesis then reappearing at E16 with its expression remaining elevated in the differentiated muscles fibers of adult mice (Bober et al. 1991). *Mrf4*$^{-/-}$ mutants were viable and fertile, with no overt skeletal muscle defect yet rib abnormalities were observed (Zhang, Behringer, and Olson 1995). These

mice had expression levels of MYOD and MYF5 comparable to wild-type mice, while myogenin expression was increased in the adults, suggesting that myogenin could compensate for loss of MRF4 to drive normal expression of muscle specific genes. All together, these results using knockout mouse models indicate that MYOD and MYF5 are important for the commitment of cells to the myogenic lineage and maintenance of myoblasts while myogenin has a crucial role in differentiation. Additionally, MRF4 determines muscle identity in the absence of MYF5 and MYOD; this factor also being important in terminal differentiation acting downstream of myogenin (Kassar-Duchossoy et al. 2004; Hasty et al. 1993). Although forced expression of MRF4 converted non-muscle cells to myoblasts, loss of MRF4 did not significantly affect the expression of muscle specific genes for which elevated myogenin levels was able to compensate.

1.3 Myoblast Fusion

Myoblast fusion is an important step in the formation of functional myotubes in culture and myofibers *in vivo* (skeletal embryogenesis and regeneration). However, the proteins involved specifically in the process of myoblast fusion are largely unknown in mammals. The fusion process involves the migration, adhesion, cytoskeletal assembly and subsequent fusion of individual myocytes to each other or to existing myotubes, requiring multiple pathways and diverse protein families (reviewed in Hindi, Tajrishi, & Kumar, 2013).

Recent work, using targeted deletion in mice, has suggested essential roles for N-WASp, small G proteins RAC1 and CDC42, and activator of small G proteins DOCK1 in cytoskeletal assembly (Gruenbaum-Cohen et al. 2012; Vasyutina et al. 2009; Laurin et al. 2008). Indeed, mutant animals lacking function of any one of these proteins had severely compromised myofiber formation demonstrated by short and thin myofibers *in vivo*. Furthermore, primary myoblasts harvested from these mutants differentiate in culture but fail to fuse, remaining mostly

mononucleated after multiple days (Gruenbaum-Cohen et al. 2012; Vasyutina et al. 2009; Laurin et al. 2008). Myoblast fusion has also been shown to be dependent on calcium signalling, including a role for the calcium-dependent adhesion molecules M- and N-cadherin in the activation of RAC1 and RhoA-dependent β-catenin (Charrasse 2005; Charrasse et al. 2002). Furthermore, loss of *Nfatc2*, a transcription factor activated by calcium, or inhibition of calpain, a calcium-dependent protease, in myoblasts leads to decreased fusion (Barnoy, Glasner, and Kosower 1996; Pavlath and Horsley 2003; Buffolo et al. 2015). This factor positively regulates the secreted cytokine IL-4, which acts as a myoblast recruitment factor to increase cell addition during fusion (Horsley et al. 2003). Loss of IL-4 in mice results in smaller fibers, with less myonuclei compared to controls. Calcium-dependent phospholipid binding proteins Annexin A1 and Annexin A5 have been shown to be positive regulators of fusion as loss of either one inhibited myotube formation *in vitro* (Leikina et al. 2015). Membrane receptors CXCR4 and CD164 have also been shown to associate together and regulate migration and promote fusion of C2C12 myoblasts (Bae et al. 2008). Additionally, the transcription factors KLF2 and KLF4 have been shown to positively regulate C2C12 myoblast fusion as part of downstream effectors of the ERK5 (MAPK) pathway by activating the adhesion protein nephronectin; without influencing the differentiation program downstream of the myogenic regulatory factors (Sunadome et al. 2011).

Interestingly, none of the proteins mentioned above are specific to skeletal muscle. The transmembrane protein TMEM8C also known as myomaker, was the first muscle-specific fusogenic protein discovered (Millay et al. 2013). It is expressed in the myotome section of the somites, later on in the limb buds and muscles of trunk and head, and also during regeneration. *Myomaker*$^{-/-}$ animals die perinatally with the absence of mature muscle fibers although the

migration, specification and differentiation of skeletal muscle was not affected. Indeed, loss of *myomaker* results in a blockade of fusion with the majority of cells remaining mononucleated in culture while myomaker overexpression in myoblasts increases the fusion index, affecting myotube size. Moreover, myomaker expression in fibroblasts, a cell with no fusogenic activity, induces their fusion with C2C12 cells, suggesting myomaker is a muscle specific protein necessary for myoblast fusion (Millay et al. 2013). Interestingly, fibroblasts expressing myomaker could not fuse to one another, suggesting other fusion proteins were needed to confer fusogenic activity in non-muscle cells. Indeed, 3 independent groups in 2017 discovered a microprotein encoded by the gene *GM732*, now known as myomerger/minion/myomixer, which when expressed in fibroblasts along with myomaker, induced fusion between these cells (Zhang et al. 2017; Quinn et al. 2017; Bi et al. 2017). Primary myoblasts isolated from *myomerger-minion*$^{-/-}$ mice did not readily fuse in culture and remained mostly mononucleated.

1.4 PAX7 and muscle regeneration

Mature myofibers in the adult are maintained by adult muscle stem cells termed satellite cells. Quiescent, activated satellite cells and proliferating myoblasts express the paired-box transcription factor Paired box protein-7 (PAX7) (Seale et al. 2000). These PAX7 positive satellite cells can be first observed at embryonic day 16.5 (E16.5), after the formation of the basal lamina, and account for 30-35% of total muscle nuclei at birth until post-natal day 21 (P21); decreasing to less than 5% in adult mice (Seale et al. 2000; Ontell et al. 1984; Schultz 1996). Inactivation of *Pax7* in mice results in post-natal lethality at approximately 2 weeks of age with *Pax7*$^{-/-}$ animals being 50% smaller than their littermates (Mansouri et al. 1996; Seale et al. 2000). Furthermore, while *Pax7*$^{-/-}$ mutants had normal *Myf5* and *MyoD* expression during development and differentiated muscle did not show disorganization, their muscles were

completely devoid of satellite cells and no myoblasts could be observed in culture. These results strongly suggest that PAX7 is required for the specification of satellite cells to the myogenic lineage (Seale et al. 2000). Further studies using a different murine genetic background, allowing for the survival of a small number of *Pax7*$^{-/-}$ mutants into adulthood, revealed impaired regeneration after acute injury and deficient muscle growth, due to the absence of functional satellite cells (Kuang et al. 2006). Inactivation of *Pax7* in adult stem cells resulted in two different phenotypes. To this end, a conditional targeting strategy was used to excise *Pax7* exclusively in satellite cells, using a Cre-loxP and tamoxifen system coupled with lineage tracing, and showed unexpectedly that mutant satellite cells were functional and contributed to repair after an acute injury (Lepper, Conway, and Fan 2009). These results were in contrast to another study that demonstrated, using the same mutant *Pax7* allele, increased fibrosis and adipocyte accumulation leading to impaired regeneration, which was aggravated by a second injury (von Maltzahn et al. 2013). It was subsequently suggested that a small subset of cells which escaped tamoxifen-induced excision, could explain the differences between regeneration phenotypes, as the latter group used a tamoxifen containing diet for their mice to ensure maximal excision (von Maltzahn et al. 2013).

Upon muscle stimuli such as injury, most quiescent satellite cells become activated, proliferate as myoblasts, exit the cell cycle, and differentiate into myocytes to contribute to repair, while a small proportion of these satellite cells self-renew and repopulate the niche (Olguin and Olwin 2004; Zammit 2002; Zammit et al. 2004; Kuang et al. 2007). Indeed, 3 populations of satellite-cell derived myoblasts can be identified in culture by the expression of PAX7 or MYOD. The most abundant population, PAX7-MYOD+ myoblasts (59.5%), are destined for differentiation; PAX7+MYOD- (23.2%) myoblasts are quiescent and will return to

the satellite cell niche; while PAX7+MYOD+ (17.3%) are proliferating myoblasts and can downregulate either PAX7 or MYOD through asymmetrical division to create a self-renewing or differentiating cell (Zammit et al. 2004). Lineage tracing experiments revealed that 90% of satellite cells have expressed *Myf5* at one point, while 10% have never expressed *Myf5* (Kuang et al. 2007). It was postulated that the small population of cells that have never expressed *Myf5* divide asymmetrically to give rise to 2 cells with differential MYF5 expression; PAX7+MYF5- cells will self-renew while PAX7+MYF5+ will proliferate and a proportion will differentiate (Kuang et al. 2007). Figure 1 shows sequential expression of PAX7 in satellite cells and induction of the myogenic regulatory factors during myogenesis.

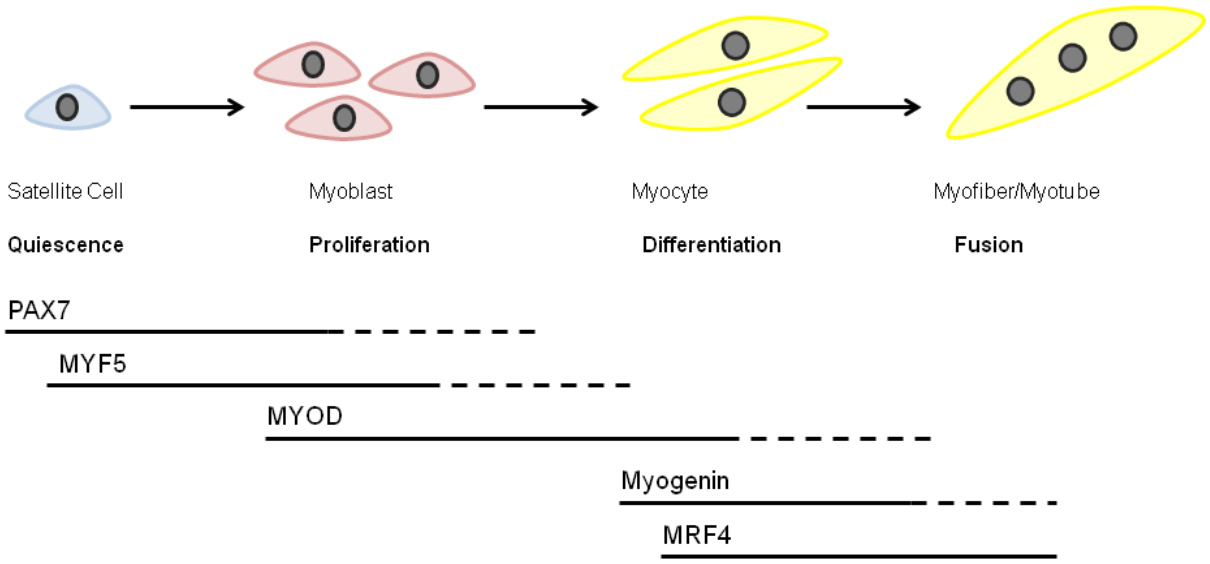

Figure 1. Myogenic regulatory factors regulate myogenesis. Quiescent satellite cells (blue) express the transcription factor PAX7 and the myogenic regulatory factor (MRF) MYF5. Upon stimuli such as exercise or injury, satellite cells become activated and proliferate. Proliferating myoblasts (red) express PAX7 and the myogenic regulatory factors (MRFs) MYOD and MYF5. A population of these myoblasts will maintain MYOD expression, downregulate PAX7 and MYF5 and start expressing myogenin and the terminal differentiation factor MRF4, as they differentiate into myocytes. Self-renewing cells will downregulate MYOD while still expressing PAX7 and return to mitotic quiescence. Differentiated myocytes will subsequently fuse to each other in culture to form multinucleated myotubes or fuse to existing myofibers *in vivo*.

1.5 Transforming Growth Factor Beta Signalling

The Transforming Growth Factor beta (TGFβ) signalling pathway is involved in many cellular processes in the adult and embryo, including roles in cell proliferation, differentiation, inflammation, wound healing, and apoptosis. TGFβ signalling is also a potent inhibitor of myogenesis and regulates muscle stem cell function. At first view, the TGFβ pathway might seem simplistic; upon ligand binding to the receptor complex, the receptors activate transcription factors, which in turn gain access to the nucleus to bind DNA and activate transcription of target genes. Far from being simple, the TGFβ super-family is made up of 30 related members that act as ligands, multiple trans-membrane receptors, numerous effector proteins (SMADs 1-8), and many more accessory binding proteins which act to generate the biological effects of this pathway. Moreover, while almost every cell in the body is responsive to TGFβ family signals, the biological effects appear to be cell type and context-dependent. There are 3 TGFβ isoforms: TGFβ1, TGFβ2 and TGFβ3 and, for the purpose of this thesis, TGFβ will refer to TGFβ1, the most prevalent isoform.

1.6 Discovery of TGFβ and its receptors

TGFβ, first described as sarcoma growth factor (SGF), was characterized by De Larco and Todaro in 1978, as a growth factor found in the conditioned medium of mouse fibroblasts infected with sarcoma virus (de Larco and Todaro 1978). SGF, which consisted of 3 distinct proteins, was distinct from epithelial growth factor (EGF) but had competing activity for the EGF receptor, promoted the formation of colonies and induced cells to grow in soft agar, a property usually reserved for cancerous cells (Roberts et al. 1981; de Larco and Todaro 1978). In 1982, TGFβ was separated from other components, TGFα and EGF, and the cell surface receptor was identified (Roberts et al. 1982). Subsequently, 3 TGFβ receptors were discovered: TGFβ-

receptor I (TGFβRI), TGFβ-receptor 2 (TGFβR2), and TGFβ-receptor 3 (betaglycan), alongside other receptors with affinity to TGFβ and other TGFβ family members, including activin and inhibin (Boyd and Massague 1989; Massagué and Like 1985; Cheifetz, Like, and Massagué 1986; Kawabata, Chytil, and Moses 1995).

The structure of the TGFβ receptors can be divided into 3 main regions: an extracellular ligand binding domain at the N-terminus, a transmembrane region and a serine/threonine kinase domain at the C-terminus (reviewed in De Caestecker, 2004; Lin & Moustakas, 1994). TGFβ signals are mediated by the receptor SMAD (R-SMAD) family of transcription factors. Briefly, SMAD2 and SMAD3 are the signal transducers for TGFβ/activin/myostatin signalling, while SMAD1/5/8 mediates the downstream signals for Bone Morphogenetic Protein (BMP) signalling (Hoodless et al., 1996; Massagué, Seoane, & Wotton, 2005; Nakao et al., 1997). The common mediator SMAD, SMAD4, associates with receptor SMADs to promote their translocation to the nucleus (Feng et al. 1998; Lagna et al. 1996). Lastly, SMAD6 and SMAD7 are inhibitory SMADs as they antagonize the activity of R-SMAD/SMAD4 complexes by interacting with the TGFβ-type I receptor, interfering with SMAD-SMAD interactions and repressing target genes (Ishida et al. 2000; Bai et al. 2000; Hayashi et al. 1997). This thesis will focus on SMAD2 and SMAD3 and their role in myogenic differentiation.

1.7 Functions of TGFβ

TGFβ was shown to have anti-proliferative effects in several cell types including epithelial, endothelial and hematopoietic cells and therefore was classified as a tumour suppressor gene (reviewed in Kubiczkova, Sedlarikova, Hajek, & Sevcikova, 2012; Moustakas, Pardali, Gaal, & Heldin, 2002). TGFβ exerted its effects on target cells mainly during G1 phase of the cell cycle, where it increased the expression of the cyclin-dependent kinase (CDK) inhibitors p15 and p27

and inhibited c-Myc and CDKs, leading to growth inhibition (Robson, 1999). TGFβ can also stimulate the differentiation of mesenchymal cell types including fibroblasts and osteoblasts (Roelen and Dijke 2003). Persistent TGFβ expression in skeletal muscle leads to the transformation of myogenic cells into fibrotic cells after injury, causing fibrosis (Y. Li et al. 2004). The roles of the 3 TGFβ isoforms was investigated *in vivo* and revealed non-compensatory roles (Sanford et al. 1997; Koo et al. 2001; Larsson et al. 2001). Of interest, targeted deletion of TGFβ1, TGFβ-receptor type I or TGFβ-receptor type II revealed a crucial role for these proteins in regulating inflammation and immune responses (Levéen et al. 2002; Larsson et al. 2001; Christ et al. 1994). Specifically, although disruption of TGFβ1 in mice yielded no developmental defects, these animals died around 20 days after birth from a wasting syndrome and generalized inflammatory response, suggesting a role for TGFβ1 in suppressing immune cell proliferation and activity of pro-inflammatory cytokines (Kulkarni et al. 1993; Shull et al. 1992). TGFβ1 and TGFβ2 are upregulated after injury and in wound healing; TGFβ1 has a prominent role in extracellular matrix remodeling, while TGFβ3 is thought to promote scar-free healing (Occleston et al. 2011; Chang et al. 2014; Roberts et al. 1986).

1.8 TGFβ signalling cascade

The TGFβ signalling cascade starts when dimerized TGFβ ligands bind to the TGFβ receptor Type II found on almost all cell types (J Massagué 1998). This TGFβ Type II receptor, which has constitutively active kinase activity, recruits the TGFβ Type I receptor (also known as ALK5) and activates it by phosphorylation at its cytoplasmic glycine/serine (GS) domain leading to the subsequent activation of the TGFβ signalling effector proteins, the receptor-SMADs (R-SMADs) (Wrana et al. 1994). Both Type I and Type II TGFβ receptors are needed for SMAD2 and SMAD3 activation. SMAD2 and SMAD3 are the signal transducers for

TGFβ/activin/myostatin signalling while SMAD1/5/8 mediate the downstream signals for Bone Morphogenetic Protein (BMP) signalling (Nakao et al. 1997; Chen, Bhushan, and Vale 1997; Hoodless et al. 1996; Yamamoto et al. 1997; Nishimura et al. 1998). Phosphorylated R-SMADs associate with SMAD4 and translocate into the nucleus to act on their target genes (Figure 2).

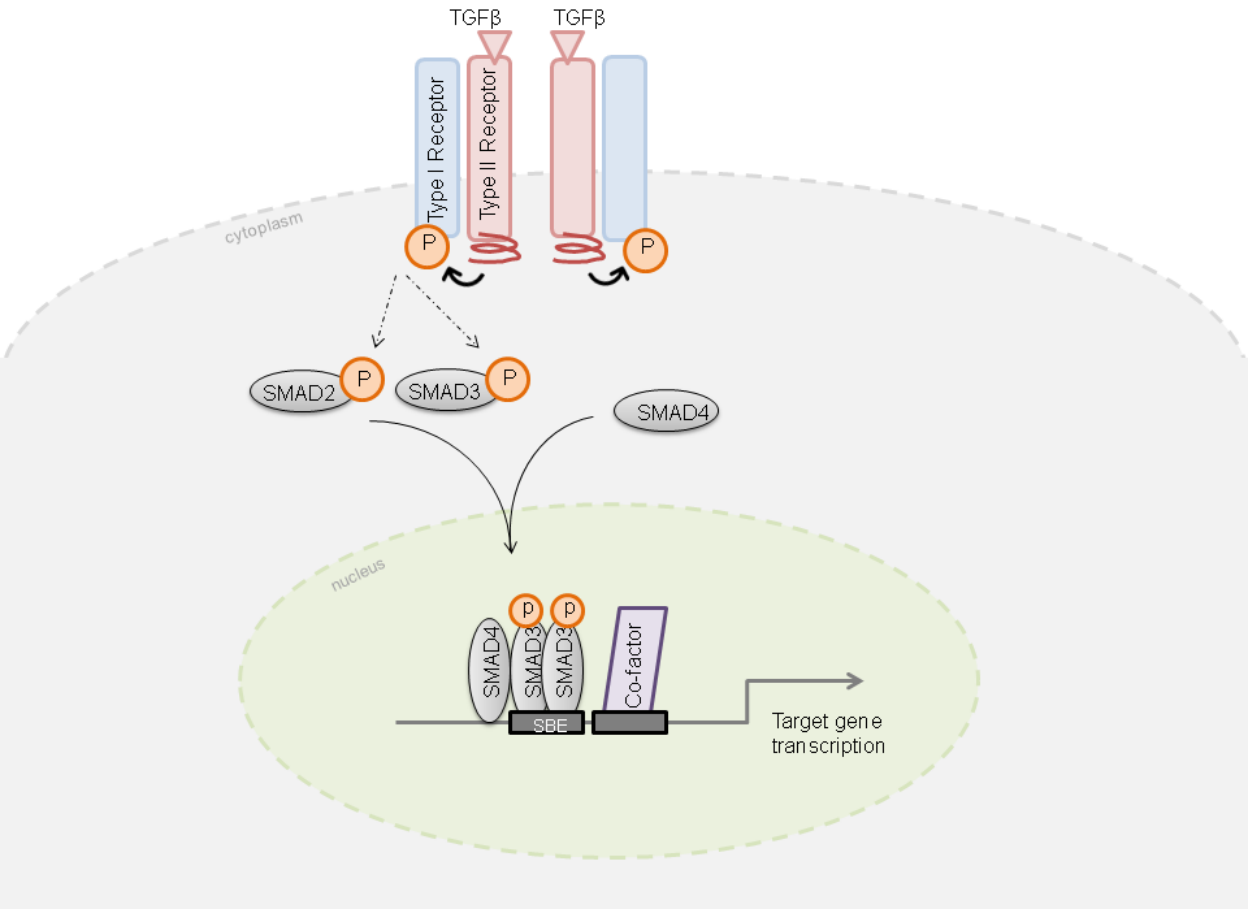

Figure 2. Classical TGFβ signalling pathway. The canonical signalling cascade begins when TGFβ ligands bind to the TGFβ Type II receptor which is constitutively active, leading to recruitment of the TGFβ Type I receptor. Subsequently, the Type I receptor is phosphorylated, becomes active and phosphorylates SMAD2 and SMAD3 at their C-terminal SSXS motif. Upon phosphorylation, SMAD2 and SMAD3 interact with SMAD4 and translocate to the nucleus to activate or repress target genes via DNA binding (indirectly for SMAD2) at Smad Binding Elements (SBE) found in promoter regions.

SMAD2 and SMAD3 contain 3 distinct regions; 2 MAD homology domains (the N-terminal MH1 domain and the C-terminal MH2 domain) separated by a less well conserved linker region (Kretzschmar and Massagué 1998; Massagué 1998) (Figure 3). The DNA-binding domain (DBD) of SMAD2/3 is located in the MH1 domain, taking the form of a β-hairpin, and mediates DNA binding and direct interaction with transcription factors, although SMAD2 is unable to bind DNA directly (Shi et al. 1998; Dennler, Huet, and Gauthier 1999). The MH1 domain also contains a nuclear localization-like signal (NLS) for direct contact with the nuclear pore complex and mediates interaction with transcription factors (Xiao et al. 2000; Wu et al. 1997; Zhang, Musci, and Derynck 1997). The main feature of the C-terminal MH2 domain is the SSXS motif, which contains the 2 serine residues essential for activation by the TGFβ Type I receptor (Ser423/Ser425 for SMAD3 and Ser465/Ser467 for SMAD2). Upstream of the SSXS motif is the L3 loop, a conserved region in SMADs which confers receptor specificity (Lo et al. 1998). The MH2 domain also mediates interactions with cytoplasmic proteins, the co-SMAD SMAD4, and DNA-binding co-factors. One such cytoplasmic protein, SMAD anchor for receptor activation (SARA), is responsible for presenting single SMAD proteins to the TGFβ Type I receptor for phosphorylation (Tsukazaki et al. 1998). Additionally, the MH1 domain retains the SMADs in the cytoplasm by binding to the MH2 domain. Upon TGFβ ligand binding, SMADs are phosphorylated at their C-terminal tails by the TGFβ Type I receptor resulting in their release from SARA, formation of complexes with SMAD4 and nuclear translocation to act on target genes (Abdollah et al. 1997; Macías-Silva et al. 1996; Souchelnytskyi et al. 1997; Tsukazaki et al. 1998). Nucleo-cytoplasmic shuttling of SMADs appears to be dynamic even in the absence of TGFβ signalling, through direct contact with the nuclear pore; although maximal SMAD accumulation occurs upon activation by TGFβ (Hill 2009). Once in the nucleus, the MH1

domain of SMAD3 makes contact with DNA (SMAD2 has transcriptional activity through SMAD4 DNA binding) by binding the consensus sequence CAGAC, along with DNA-binding co-factors which stabilize the SMAD complexes on DNA (Dennler, Huet, and Gauthier 1999).

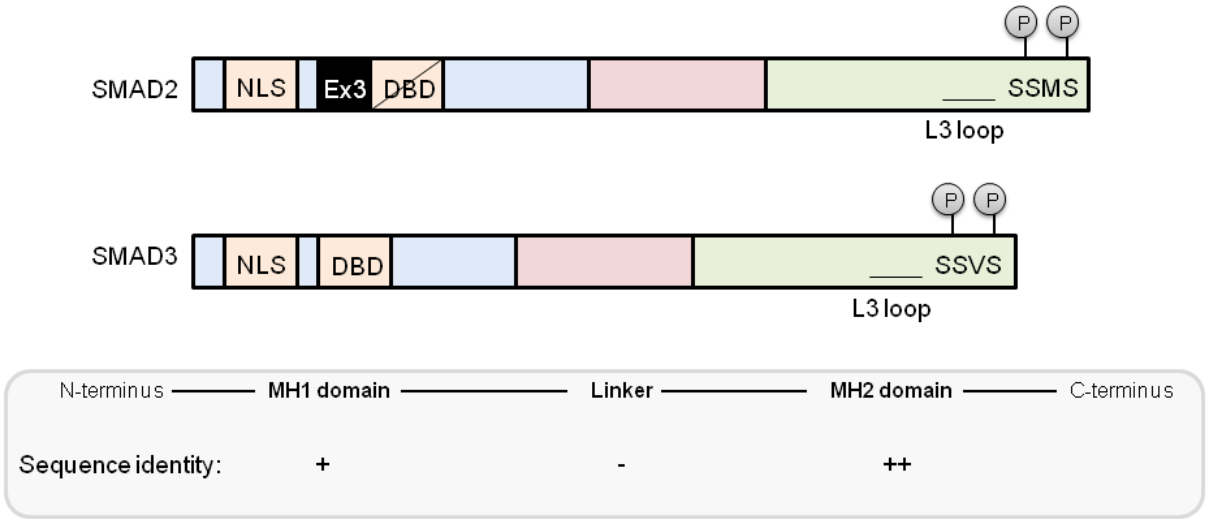

Figure 3. General protein structure of SMAD2 and SMAD3. R-Smads are organized into 3 domains: the MH1 domain found in the N-terminal portion of the protein (blue), the MH2 domain (green) found in the C-terminal part of the protein and the centrally located linker region (red) which has low sequence identity among members and is a site for post-translational modifications. The C-terminal tail includes a SSXS motif in which the second and third serines are phosphorylated by the TGFβ-receptor upon ligand binding. The L3 loop adjacent to the SSXS motif is conserved in SMADs and is important for receptor specificity. SMAD2 contains an extra region, encoded by exon 3 (black rectangle), situated directly before the β-hairpin DNA-binding motif (DNA Binding domain -DBD, in orange) that is not present in SMAD3 and prevents SMAD2 from binding DNA directly. The nuclear localization signal (NLS) is shown upstream of the DBD at the N-terminus of SMAD2 and SMAD3.

Given that: i) SMAD binding elements (SBE, 5'-CAGAC-3') are plentiful in the genome and SMAD2 also binds to GC rich sequences, and ii) SMAD2 and SMAD3 are weak DNA binders, SMAD binding to these sequences alone would not confer great specificity or control over target gene expression. Indeed, SMAD2 and SMAD3 complexes need co-activators and repressors to anchor DNA and regulate gene transcription, including the transcriptional co-activators p300, CBP and P/CAF (Simonsson et al. 2006; Pouponnot, Jayaraman, and Massagué 1998; Itoh et al. 2000; Janknecht, Wells, and Hunter 1998). One such co-factor, FAST1, interacts with SMAD2/4 and SMAD3/4 complexes to regulate target genes, whereas BMP signalling-effector SMADs do not (Yeo, Chen, and Whitman 1999). Furthermore, SMAD2/FAST2 complexes activate the TGFβ-responsive *goosecoid* promoter (Labbé et al. 1998). Replacing SMAD2 by SMAD3 in this complex had an inhibitory effect on the promoter, suggesting that SMAD2 and SMAD3 can have different functions depending on the associated co-factor (Labbé et al. 1998).

Even though SMAD2 and SMAD3 are very similar in structure, are activated by the same receptor and bind the same DNA response element in target promoters, a growing number of studies suggest that these transcription factors are not redundant in function. In the kidney, where TGFβ induces tubulo-interstitial fibrosis, SMAD3 and SMAD2 regulate a different subset of genes (Meng et al. 2010; Phanish et al. 2006). In pancreatic ductal adenocarcinoma, SMAD2 and SMAD3 also have differential roles in relaying TGFβ signals; SMAD3 promotes growth inhibition, while SMAD2 enhances migration (Ungefroren et al. 2011). Moreover, in primed pluripotent cells, SMAD3 is dispensable for the maintenance of the undifferentiated state, while SMAD2 expression is necessary (Sakaki-Yumoto et al. 2013). In fact, TGFβ can inhibit the expression of SMAD3 further suggesting that SMAD3 has functions that are not regulated by

TGFβ and can counteract SMAD2 activities in some systems (Baugé et al. 2011). Further, experiments in the chick embryo revealed that SMAD2 and SMAD3 could cooperate but also had opposing roles in neurogenesis. Forced expression of SMAD3 alone in neural progenitors enhanced migration and differentiation of these cells, leading to productive neurogenesis while addition of both SMAD2 and SMAD3 had a synergistic effect (Miguez et al. 2013). Interestingly, knockdown of SMAD2 and SMAD3 had different outcomes in this context; loss of SMAD2 increased while loss of SMAD3 decreased neurogenesis, revealing a complex interplay where SMAD2 can cooperate with SMAD3 or antagonize its actions (Miguez et al. 2013).

1.9 Knockout mouse models of SMAD2 and SMAD3

To further characterize the functions of SMAD2 and SMAD3 *in vivo*, knockout-mouse models were created with important phenotypic differences. Two separate groups produced a *Smad3*-knockout mouse model, using a targeted deletion of exon 2 and exon 8 respectively. *Smad3*$^{ex2/ex2}$ mutant mice were viable and fertile, although they were approximately 30% smaller in size compared to control littermates (Zhu et al. 1998). These mice had an increased propensity to develop colorectal tumours and metastatic adenocarcinomas, consistent with a role for SMAD3 as a tumour suppressor (Zhu et al. 1998).

Smad3$^{ex8/ex8}$ mutants were generated by introducing a premature stop codon directly after exon 7, producing a truncated SMAD3 lacking the last 89 amino acids that contains the two serine residues (SSVS motif) required for activation by the TGFβ Type I receptor (Yang 1999). *Smad3*$^{ex8/ex8}$ mutants were born at Mendelian ratios and were smaller than controls. In contrast, although *Smad3*$^{ex8/ex8}$ mutants did not develop adenocarcinomas, the majority died before 3 months of age due to immune dysregulation and chronic infections (Yang 1999).

Yet another *Smad3*$^{-/-}$ mouse model was used to study involvement of SMAD3 in TGFβ-mediated inhibition of cell proliferation. In these animals, *Smad3* transcription was halted by targeting exon 1, containing the ATG start codon and part of the first intron (Datto et al. 1999). Consistent with the other two phenotypes, *Smad3*$^{-/-}$ mice showed no embryonic or perinatal lethality, were smaller in size and had some limb defects. *Smad3*$^{-/-}$ fibroblasts taken from these animals had increased proliferation and were insensitive to inhibition of cell proliferation by TGFβ, supporting a role for SMAD3 in TGFβ-mediated homeostatic control of immune cells (Datto et al. 1999). In sharp contrast to the different *Smad3*$^{-/-}$ phenotypes, deletion of *Smad2* is embryonic lethal. Indeed, three *Smad2*$^{-/-}$ mutants, with targeted disruptions in either the MH1 domain (Nomura and Li 1998; Waldrip et al. 1998) or MH2 domain (Weinstein et al. 1998), displayed lethality early in embryonic development; these embryos failed to gastrulate and induce mesoderm. As expected, *Smad4*-/- mutants also die early in embryonic development and fail to undergo gastrulation to induce mesoderm formation (Yang et al. 1998; Sirard et al. 1998).

SMAD2 is alternatively spliced to give rise to two isoforms including the full length SMAD2 that is 467 amino acids in length, and a shorter SMAD2 isoform, lacking exon 3 in its N-terminal MH1 domain (SMAD2ΔEx3) of only 427 amino acids (Yagi et al. 1999; Takenoshita et al. 1998). Interestingly, despite high conservation between SMAD2 and SMAD3, SMAD3 does not contain the sequence encoded by SMAD2 exon 3. This extra exon has been linked to the inability of SMAD2 to bind DNA directly or to interact with other proteins through its MH1 domain in the cytoplasm (Dennler, Huet, and Gauthier 1999). Indeed, experiments using TGFβ responsive promoters, such as the Plasminogen Activator Inhibitor type 1 (PAI-1), revealed that a SMAD2 mutant, lacking the amino acids encoded by exon 3, had transcriptional activity by itself, unlike the full length SMAD2 (Dennler, Huet, and Gauthier 1999). Indeed, loss of exon 3

through alternative splicing renders SMAD2ΔEx3 the ability to bind DNA and regulate transcription (Dennler, Huet, and Gauthier 1999). Interestingly, mice expressing only the short SMAD2 isoform are viable and capable of activating all important TGFβ target genes, suggesting that the full length isoform is dispensable for embryonic development (Dunn et al. 2005). The role of the short SMAD2 isoform remains unknown, though it is expressed in skeletal muscle, heart and placenta (Takenoshita et al. 1998).

1.10 TGFβ in myogenesis

During myogenesis, treatment with TGFβ is known to potently inhibit the differentiation and fusion process (Liu, Black, and Derynck 2001; Liu, Kang, and Derynck 2004; J Massagué et al. 1986; Olson et al. 1986). TGFβ-induced inhibition of myogenesis is only effective early in differentiation and is reversible when cells are switched to TGFβ-free medium (J Massagué et al. 1986; Olson et al. 1986). TGFβ treatment inhibits myogenic differentiation through activation of SMAD3, but not SMAD2, which interferes with the formation of muscle regulatory factor-containing transcriptional complexes (Liu, Kang, and Derynck 2004; Liu, Black, and Derynck 2001). Specifically, SMAD3 physically interacts with MYOD's bHLH domain to block interaction of MYOD with E-proteins and thereby binding of E-boxes on muscle-specific promoters (Liu, Black, and Derynck 2001). SMAD3 also acts to inhibit MYOD-dependent transcription through inactivation of MEF2C, a myogenic co-regulator highly expressed in skeletal muscle (Liu, Kang, and Derynck 2004; Molkentin et al. 1995). In both cases, this repression by SMAD3 did not involve contact with DNA and was instead mediated through its C-terminal MH2 domain. The role of SMAD2 in the TGFβ-mediated inhibition of myogenic differentiation is less well understood as SMAD2 was not found to interact with MEF2C in the Liu, 2004 study.

The muscle phenotype of a SMAD3 knockout mouse, which lacked exon 8 coding for the C-terminal SSVS motif (Yang, 1999); described above), was found not to be consistent with a negative role in myogenic differentiation (Ge et al. 2011; Ge et al. 2012). Muscle characterization of these SMAD3 mutants revealed a decrease in average myofiber cross-sectional area (XSA), reduced myoblast proliferation and defective myogenic differentiation consistent with a decrease in cell cycle genes and myogenic gene expression (Ge et al. 2011). Furthermore, in-culture and myofiber experiments revealed a decreased percentage of quiescent MYOD-/PAX7+ satellite cells and lowered overall satellite cell numbers. Muscle regeneration after acute injury was also impaired in the *Smad3* knockout mice (Ge et al. 2012). Indeed, these animals had a defective inflammatory response consistent with a smaller number of invading mononuclear cells one day after injury, leading to decreased regenerated fiber size (Ge et al. 2012). These results were surprising as TGFβ was suggested to negatively affect muscle regeneration by inhibiting satellite cell proliferation, fusion and expression of muscle-specific genes (Allen and Boxhorn 1987; Olson et al. 1986; Massagué et al. 1986). In line with a positive role for SMAD3 in the regulation of myogenesis, recent studies have demonstrated that master transcription factors, which regulate cell identity and maintenance, such as OCT4 in embryonic stem cells and MYOD in myotubes, recruit SMAD3 to their cell-specific targets (Mullen et al. 2011). Indeed, this work suggested that the recruitment of SMAD3 to myogenic promoters by MYOD was necessary for efficient differentiation. Loss of MYOD in myotubes lead to decreased SMAD3 occupancy of genes usually co-occupied with MYOD, resulting in defective differentiation. Moreover, forced expression of a master transcription factor in a different cell type, such as MYOD in mouse embryonic stem cells, resulted in the recruitment of SMAD3 to new MYOD targets, while still occupying its original sites with OCT4 (Mullen et al. 2011).

These findings suggest that SMAD3 assumes a pro-myogenic role in myogenesis, in sharp contrast to the potently anti-myogenic role of TGFβ signalling. In contrast to advances in understanding the role of SMAD3 in myogenesis, little is known about the contribution of SMAD2 to this process.

1.11 CCAAT/Enhancer Binding Proteins

Our laboratory is interested in the function of the transcription factor CCAAT/Enhancer Binding Protein Beta (C/EBPβ) in myogenesis and satellite cell maintenance. C/EBPβ is part of the CCAAT/Enhancer Binding Protein (C/EBP) family comprised of 6 transcription factors with roles in cell proliferation, differentiation, immune regulation and inflammation (Ramji and Foka 2002). Named for their ability to bind CCAAT boxes and enhancer elements in promoter regions of target genes, the C/EBP proteins bind DNA in the form of homodimers or heterodimers between family members, by way of their basic leucine zipper (bZIP) domain. This highly conserved bZIP domain is located at the C-terminal tail and is responsible for DNA binding and protein-protein interaction, while the transactivation domain, found at the N-terminal portion of the protein, is necessary for interaction with the basal transcription machinery and thereby the activation of transcription.

C/EBPα, the first characterized C/EBP family member, was cloned from rat liver and found to be highly expressed in terminally differentiated cells from the liver and adipose tissue (Landschulz, Johnson, and McKnight 1988). Following the discovery of C/EBPα, numerous studies simultaneously identified a distinct but highly related C/EBP family member (Akira et al. 1990; Descombes et al. 1990; Poli, Mancini, and Cortese 1990; C. J. Chang et al. 1990; Roman et al. 1990). This protein was identified as nuclear factor IL-6 (NF-IL6) for its ability to regulate IL-6, an important cytokine in the immune response and independently as Liver-enriched

transcription activator protein (LAP), a factor with 71% sequence homology with C/EBPα in the bZIP domain (Descombes et al. 1990). NF-IL6 and LAP were found to in fact be the same protein and were renamed C/EBPβ to maintain consistent nomenclature (Cao, Umek, and McKnight 1991). *Cebpb* is an intronless gene whose mRNA produces 3 different protein isoforms due to leaky ribosome scanning (Cao, Umek, and McKnight 1991). These C/EBPβ isoforms, LAP*, LAP and LIP (Liver-enriched inhibitory protein), have different molecular weights (38, 35 and 20 kDa respectively) due to N-terminal truncations and have different ratios of tissue expression. LAP*, the full length isoform and LAP, the most widely expressed isoform that lacks the first 21 amino acids present in LAP*, both contain the bZIP and transactivation domain and are transcriptional activators. LIP, the smallest of the 3 isoforms, can interact with LAP* and LAP via an intact bZIP domain, but because LIP lacks a transactivation domain, it is a dominant negative protein, inhibiting the functions of LAP* and LAP.

1.12 C/EBPβ function in myogenesis

C/EBPβ is known as a regulator of mesenchymal stem cell fate, however little is known about its role in skeletal myogenesis. The muscle phenotype of the *Cebpb*$^{-/-}$ animals was investigated and revealed normal skeletal muscle histology; however enhanced insulin sensitivity was associated with loss of *Cebpb* (Wang et al. 2000). Furthermore, loss of *Cebpb* in macrophages resulted in impaired muscle regeneration after injury (Ruffell et al. 2009).

Our laboratory has found that C/EBPβ is co-expressed with PAX7 in cultured satellite cell-derived myoblasts and in healthy muscle (Marchildon et al. 2012). We found that C/EBPβ is highly expressed by myoblasts in growth conditions and is rapidly reduced upon induction to differentiate, paralleled by the loss of PAX7 expression, and upregulation of myogenin and MyHC (Marchildon et al. 2012). Forced expression of C/EBPβ in myoblasts increased PAX7

expression and decreased MYOD protein expression, resulting in the blockade of myogenic differentiation, concomitant with decreased expression of myogenin and MyHC (Marchildon, 2012). Indeed, C/EBPβ is a direct regulator of PAX7 expression, occupying the PAX7 promoter in primary myoblasts and activating *Pax7* promoter activity (Marchildon et al. 2012).

Conditional excision of *Cebpb* in PAX7-expressing cells, using a tamoxifen-inducible CreER system to target the floxed *Cebpb* gene, resulted in precocious differentiation under growth conditions and robust fusion of myotubes in culture (Marchildon et al. 2012). Furthermore, *in utero* excision of *Cebpb* in satellite cells caused fiber hypertrophy at post-natal day 21 (P21). Moreover, after a $BaCl_2$-induced injury C/EBPβ cKO animals had enhanced regeneration featuring increased average cross sectional area compared to controls. Consistent with a role as a negative regulator in muscle, C/EBPβ expression is increased in muscle pathologies such as sarcopenia and cancer cachexia (Giresi et al. 2005; Marchildon et al. 2015)

Furthermore, our laboratory has recently shown that C/EBPβ has a positive role in satellite cell self-renewal (Lala-Tabbert et al. 2016). Indeed, loss of C/EBPβ in primary myoblasts and in myofiber-associated satellite cells resulted in differentiation at the expense of self-renewal, as demonstrated by an increase in the population of PAX7-/MYOD+ cells and a decrease in the population of PAX7+/MYOD- cells. Consequently, satellite cells lacking C/EBPβ become activated and efficiently contribute to repair a first injury, but since the satellite cell niche is not replenished with more cells diverted to differentiation, a second acute injury cannot be efficiently repaired in *Cebpb*^{cKO} mice (Lala-Tabbert et al. 2016). Additionally, consistent with a role as a novel regulator of satellite cell homeostasis, transient stimulation of C/EBPβ expression using the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX), re-programs myoblasts to a more stem cell-like state, with increased expression of satellite cell markers, improved cell

expansion in culture and enhanced niche repopulation after transplantation *in vivo* (Lala-Tabbert, Fu, and Wiper-Bergeron 2016).

1.13 Regulating C/EBPβ activity

Retinoic acid (RA) is a metabolite of vitamin A involved in many cellular processes during embryogenesis and in the adult (Niederreither and Dollé 2008). The actions of RA on target genes are mediated through binding of RA ligands to the nuclear receptors RAR (retinoic acid receptor) and RXR (retinoid X receptor), which form heterodimers and bind to RAREs (retinoic acid response elements) on DNA (Petkovich et al. 1987). RA and C/EBPβ have both been implicated in the regulation of mesenchymal stem cell fate; having different effects on differentiation into the osteoblast and adipocyte lineages (Figure 4). Indeed, RA treatment of C3H10T1/2 mouse mesenchymal stem cells induces their differentiation into osteoblasts; while ectopic expression of C/EBPβ blocks induction of osteoblastogenesis (Gazit et al. 1993; Wiper-Bergeron, St-Louis, and Lee 2007). It was demonstrated that C/EBPβ negatively regulates the expression of RUNX2, a master regulator of osteoblastogenesis, in these cells by binding to a negative response element in the *Runx2* P1 promoter (Wiper-Bergeron, St-Louis, and Lee 2007). Treatment with RA resulted in the displacement of C/EBPβ from the *Runx2* promoter, leading to increased RUNX2 expression and differentiation of osteoblasts (Wiper-Bergeron, St-Louis, and Lee 2007). This mechanism of action of RA was found to be indirect; mediated through the upregulation of SMAD3 expression which interacted with C/EBPβ (Dingwall et al. 2011).

A similar mechanism is also involved in the RA-induced inhibition of adipogenesis (Marchildon et al. 2010). In RA-treated pre-adipocytes, C/EBPβ expression is induced normally but its downstream targets C/EBPα and PPARγ are not expressed, leading to a failure in adipogenesis. Similar to the events in osteoblast differentiation, RA actions were found to be

indirect and mediated through SMAD3, which blocked C/EBPβ occupancy of the *Cebpa* promoter, a master regulator of adipogenesis. Of note, in both of these systems, treatment with RA upregulates SMAD3 expression resulting in increased nuclear SMAD3 that is not C-terminally phosphorylated, but can still bind C/EBPβ and inhibit its DNA occupancy. These results suggest that the nuclear accumulation and the protein-binding actions of SMAD3 in this context are TGFβ-independent.

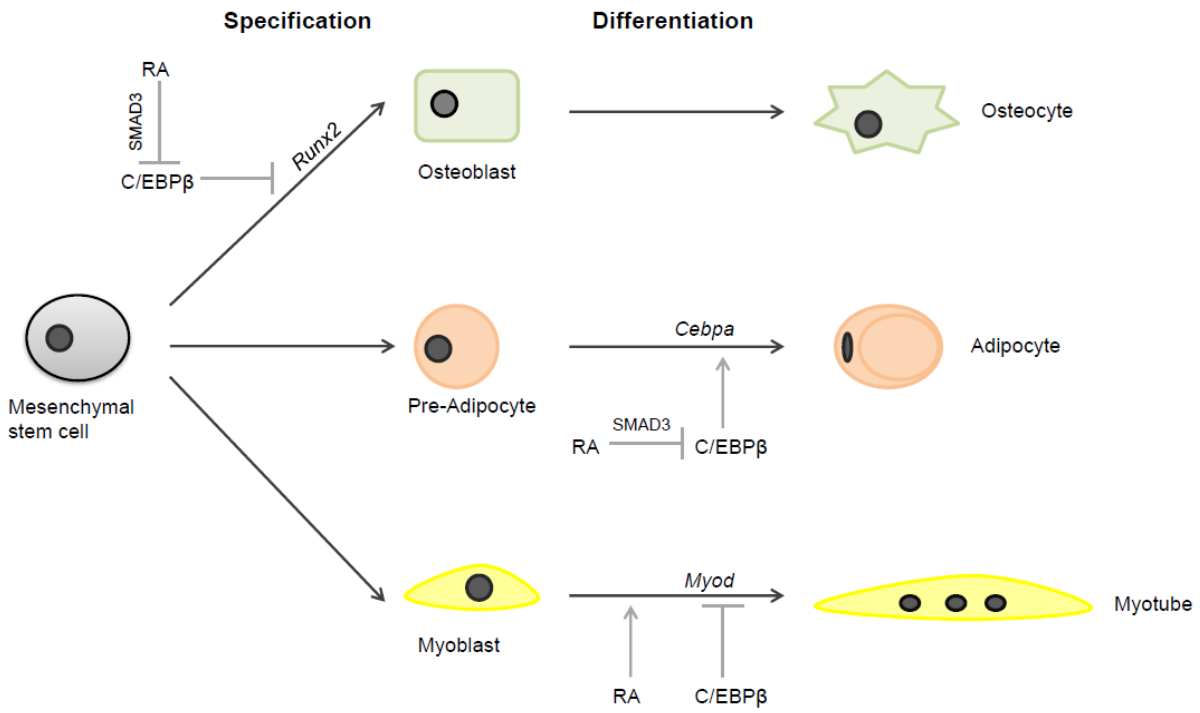

Figure 4. C/EBPβ regulates mesenchymal stem cell fate. Ectopic expression of C/EBPβ blocks osteoblast differentiation in uncommitted mesenchymal stem cells while driving adipogenesis. Retinoic acid (RA) blocks the differentiation of mesenchymal stem cells into osteoblasts by preventing C/EBPβ from binding the Runx2 promoter, where it acts as a negative regulator. In this case, RA does not act directly on C/EBPβ but through Smad3 to disrupt C/EBPβ DNA binding. In osteoblasts already committed to differentiate, C/EBPβ switches from negative to a positive regulator and promotes osteoblast differentiation. C/EBPβ drives mesenchymal stem cell differentiation into adipocytes by promoting the expression of C/EBPα, a master regulator of these cells. RA inhibits adipogenesis by upregulating Smad3 expression which in turn displaces C/EBPβ from the *Cebpa* promoter where it is a positive regulator. Ectopic expression of C/EBPβ inhibits myogenesis; TGFβ is a well known inhibitor of myogenesis whose actions are also mediated through Smad3, while retinoic acid promotes myogenesis.

1.14 Retinoic acid in myogenesis

Retinoic acid is known to inhibit proliferation and to promote differentiation in a variety of cell types including myoblasts (Edwards and McBurney 1983; Alric et al. 1998; Ryan et al. 2012; Kennedy et al. 2009; Hamade et al. 2006). RA treatment enhances the differentiation of myoblasts into large myotubes and promotes embryonic skeletal myogenesis by activating *fgf8* to induce the expression of MyoD in zebrafish (Halevy and Lerman 1993; Hamade et al. 2006). Moreover, in both P19 mouse embryonal carcinoma cells and mouse embryonic stem cells, treatment with RA enhanced myogenesis promoting the expression of the mesodermal marker *Wnt3a*, skeletal muscle progenitor markers *Pax3* and *Meox1*, and myogenic regulatory factors *MyoD1* and *Myog* (Kennedy et al. 2009). Further, RA treatment can rescue the anti-myogenic effects of TGFβ in C2C12 myoblasts (Krueger and Hoffmann 2010).

1.15 Models of myogenic differentiation

Well characterized models are available to study skeletal muscle development *in vitro* and *in vivo*. C2C12 cells are an immortalized cell line obtained by selective serial passage of myoblasts after a crush injury to muscles of a C3H mouse (Yaffe and Saxel 1977; Blau et al. 1985). C2C12 cells are a popular choice for their accessibility and ability to proliferate indefinitely and to differentiate under appropriate culture conditions. Primary myoblasts freshly isolated from mouse hindlimb muscles offer another method to study myogenesis *in vitro.* Although more laborious to harvest, purify and maintain in a proliferative state in culture for extended periods, primary myoblasts are the experimental cell of choice in the field, as they are more physiologically relevant than immortalized C2C12 cells, which are polyploid and have inactivation of the p19/Arf locus (Chang et al. 2007; Pajcini et al. 2010).

Although MRFs are expressed in both cell types during myogenic differentiation, we have observed lower expression of the satellite cells markers PAX7 and C/EBPβ in proliferating C2C12 cells compared to primary myoblasts (unpublished observations). Moreover, maturation was found to be enhanced in primary myoblasts compared to C2C12 cells, as demonstrated by increased MRF4 and MyHC levels after electrical stimulation *in vitro* (Langelaan et al. 2011). Additionally, mRNA expression of *Cdh15* (M-cadherin) and other fusion genes are downregulated in C2C12 cells compared to primary myoblasts (Grabowska et al. 2011).

Muscle regeneration experiments are commonly used as a model to study adult skeletal myogenesis *in vivo*. To this end, cardiotoxin (CTX), which damages the myofibers but not the satellite cells, is administered to the tibialis anterior muscle of mice to cause a mild injury and to activate satellite cells (Couteaux, Mira, and D'Albis 1988). Other types of injuries can be used including a chemical injury using barium chloride, or a physical injury caused by freezing or crushing the muscle. The regeneration process includes 3 phases: inflammation/degradation, tissue repair and maturation (reviewed in Musarò, 2014). First, neutrophils are mobilized to the injury site within hours, followed by pro- inflammatory macrophages, which stimulate activation and proliferation of satellite cells in the first 2 days post-injury. The tissue repair stage involves recruitment of anti-inflammatory macrophages which promote myogenic differentiation and fusion of myogenic cells to each other or existing myofibers. Extent of regeneration is typically assessed 7 days post-injury, with the quantification of cross-sectional area in regenerating muscle fibers, which contain centrally located nuclei. Muscle cross-sectional area is restored approximately 1 month post-injury with quiescent satellite cells returning to their niche between 30 and 50 days post-injury.

RATIONALE AND HYPOTHESIS

Although there is evidence indicating that TGFβ, through SMAD3, is a potent inhibitor of myogenesis, recent findings using a *Smad3* knockout mice model revealed a pro-myogenic role for SMAD3 during myogenic differentiation and regeneration. Moreover, SMAD3 can interfere with C/EBPβ transcriptional activities during osteoblast and adipocyte differentiation and C/EBPβ is a known negative regulator of myogenic differentiation. Together, these results suggest TGFβ-independent functions for SMAD3 during myogenesis. Further, while SMAD2 is expressed in myoblasts, little is known about the role of SMAD2 in muscle and no model, to date, has been used to study the role of SMAD2 in adult skeletal myogenesis.

Thus, I hypothesized that *retinoic acid treatment, through upregulation of SMAD3 expression, can interfere with C/EBPβ activities in myoblasts and thereby promote myogenic differentiation*. Additionally, *I generated a conditional knockout model to study the role of SMAD2 in adult skeletal myogenesis*.

The ***specific aims*** of this study were: **(1)** to determine the effect of RA on myogenesis and to assess the function of SMAD3 in this context; and **(2)** to create and characterize a novel conditional mouse model to study the effect of loss of *Smad2* in adult skeletal myogenesis.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell culture and primary myoblast isolation

C2C12 cells (American Type Culture Collection, Manassas, VA, USA) were maintained in growth medium (GM) containing Dulbecco's Modified Eagle Medium (DMEM 4.5g/L glucose, 110 mg/L sodium pyruvate and 584 mg/L L-glutamine) and supplemented with 10% fetal bovine serum (FBS) (Wisent, Saint-Bruno, QC, Canada). Differentiation of C2C12 into myotubes was accomplished using differentiation media (DM; low serum conditions) containing DMEM supplemented with 2% horse serum (HS) (Sigma-Aldrich, St-Louis, Missouri, USA). DM was added to 80% confluent cultures for 4 days; media was replenished every day.

Primary myoblasts were isolated from mice aged 6-8 weeks. Briefly, hindlimb muscles were dissected and minced using scissors and digested for 2 hours at 37°C in collagenase. Cells were sterile filtered through a 70µm filter and subjected to 3x5 min centrifugation at 900 g. After pre-plating for 3 hours on plastic culture plates, cell suspensions were transferred to matrigel-coated plates and maintained in DMEM containing 20% FBS, 10% HS. Primary myoblasts were replenished with 10ng/ml basic fibroblast growth factor (FGF, Peprotech Rocky Hill, New Jersey) and 2ng/ml hepatocyte growth factor (HGF, Peprotech Rocky Hill, New Jersey) everyday. To induce differentiation, 80% confluent cultures were switched to low serum conditions, consisting of DMEM supplemented with 10% HS, for 2 days. More recently, magnetic activated cell sorting (MACS Miltenyi Biotec, Bergisch Gladbach, Germany) was used as an alternative method for the isolation of satellite cells. Briefly, hindlimb muscle were dissected and digested for 2 hours as mentioned above. After digestion, cell suspensions were incubated with PE-conjugated primary antibodies against CD45 (pan-hematopoeitic cell marker), CD31 (endothelial cell marker) and Sca-1 (endothelial and interstitial cell marker) (BD Biosciences, Franklin Lakes, New Jersey, USA)for 30 minutes, followed by a 30 minute

incubation with PE-microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) in DMEM containing 2% FBS (Motohashi, Asakura, and Asakura 2014). Cell suspensions were subsequently mixed with AutoMACS running buffer and ran through a LD column attached to a magnetic board to obtain cells (negative sort). Myoblasts were seeded on matrigel (Corning, Corning, New York, USA) -coated plates and maintained in DMEM containing 20% FBS, 10% HS, and replenished with growth factors (FGF and HGF) every day. All cells were grown at 37°C with 5% CO_2 and all media used for primary myoblasts were supplemented with 1% penicillin/streptomycin.

2.2 Stable cell lines

Retrovirus was made by transfecting 40% confluent Phoenix cells (American Type Culture Collection, Manassas, VA, USA) with 10µg of plasmid DNA using $CaCl_2$, chloroquine and 2X Hepes Buffered Saline (HBS). The DNA complex was added to the cells dropwise and media was changed after 9 hours. The virus was collected 48 hours post-transfection and filtered through a 0.45µm filter. Freshly collected retrovirus was used to infect C2C12 or primary myoblasts. For C2C12 cells, 1mL virus and 4µL Polybrene (Sigma-Aldrich, St-Louis, Missouri, USA) was added to 3mL GM and left to incubate for 8 hours, after which the media was changed to normal GM. Alternatively, a second method was used for infection of primary myoblasts, as these cells are known to be difficult to transduce. This second method involved incubating the virus and Polybrene complex on the cells for 15 minutes before centrifugation at 1100g for 30 minutes; changing the media after spinning (Springer and Blau 1997). Maximal transduction efficiency of these cells can be achieved with multiple "spinfections" done at least 8 hours apart. For both methods, selection of pooled stable cell lines was achieved with appropriate antibiotic;

G418 disulfate salt solution or puromycin (both Sigma-Aldrich, St-Louis, Missouri, USA) in GM for 4-7 days.

2.3 Reagents

Retinoic acid (R&D Systems Minneapolis, Minnesota, USA) was used at a concentration on 1nM. Recombinant TGFβ1 was used at a concentration of 1-5ng/mL (R&D Systems Minneapolis, Minnesota, USA), as indicated.

2.4 Generation of conditional *Smad2* knockout mice

The *Smad2* floxed (Smad2$^{tm1.1Ebp}$ JAX stock #022074) mice were purchased from The Jackson Laboratory (Ju et al. 2006) and bred to homozygosity in *Pax7*^{CreER} mice (kindly received from the Rudnicki Lab, Ottawa Hospital Research Institute, Ottawa, Canada, Nishijo et al. 2009). The tamoxifen-inducible CreER allele was engineered by Feil et al (Feil et al. 1996). Genotyping was done using primer sequences provided by the manufacturer and standard PCR protocol using MyTaq HS Red Mix (Bioline, London, UK) to identify WT (*Smad2*$^{fl/fl}$*Pax7*$^{+/+}$) and *Smad2*^{cKO} (*Smad2*$^{fl/fl}$*Pax7*$^{CreER/+}$) experimental pairs. Animals used for experiments were approximately 6-8 weeks old. To achieve *Smad2* excision *in vivo*, WT and *Smad2*^{cKO} mice were subjected to 5 daily intraperitoneal (i.p.) injections of 1.5 mg tamoxifen (Sigma-Aldrich, St-Louis, Missouri, USA) dissolved in corn oil. *In vitro* excision of *Smad2* was achieved by adding 2 µM 4-OH tamoxifen to the culture media for 2 days. *In utero* excision of *Smad2* was done by breeding a *Smad2*$^{fl/fl}$*Pax7*$^{+/+}$ female with a *Smad2*$^{fl/fl}$*Pax7*$^{CreER/+}$ male and gavaging the pregnant female with 2.5 mg tamoxifen at embryonic day 15.5 (E15.5), when PAX7 is expressed (Lepper and Fan 2010). Pups were genotyped at post-natal day 21 (P21) to identify presence of experimental pairs. Mice were housed at the University of Ottawa with free access to water and food. All

animal work was performed following Canadian Council on Animal Care guidelines and was approved by the University of Ottawa Animal Care Committee.

2.5 Regeneration experiment using cardiotoxin injury

To achieve *Smad2* excision *in vivo*, WT and *Smad2*^{cKO} mice were subjected to 5 daily i.p. injections of 1.5 mg tamoxifen dissolved in corn oil (Sigma-Aldrich, St-Louis, Missouri, USA). One week following end of tamoxifen treatment, anesthetized mice were injured by injecting 30µl of 10µM cardiotoxin (Latoxan) into the left tibialis anterior (TA) muscle, while the right TA was left uninjured. Mice were sacrificed 7 days post-injury and both TA muscles were flash frozen with Tissue-Tek optimal cutting temperature (O.C.T.) compound (VWR, Radnor, PA, USA) in isopentane and sectioned (8 µm thick) using a Microm HM 500M cryostat.

2.6 Immunohistochemistry for PAX7 and SMAD2 on frozen muscle sections

Frozen microscope slides containing muscle sections were dehydrated at 60°C for 30 minutes before being placed in a copling jar with citrate buffer (10mM citric acid, 0.05% Tween20, pH 6.0) at 92°C for 20 minutes. Slides were cooled to room temperature before quick washes in PBS-T (phosphate buffered saline with 0.05% Tween) and permeabilization 15 minutes in PBS-T. After washing, slides were blocked in PBS-T + 5% normal donkey serum for 1 hour. Primary antibody incubation was done with SMAD2 (Abcam) and PAX3/7 (Santa Cruz Biotechnology) overnight at 4°C. Slides were washed 3 times 5 minutes in PBS-T before secondary antibody incubation with Alexa-Fluor 488 anti-mouse and Alexa-Fluor 594 anti-rabbit (Invitrogen, Carlsbad, CA, USA) for 1 hour at room temperature in the dark. After incubation, slides were washed 3 times 5 minutes with PBS-T, stained with DAPI (0.5 µg/ul) for 5 minutes, rinsed 3 times 5 minutes with PBS and mounted with a coverslip with Vector Shield (Vector Laboratories Burlingame, CA, USA). Pictures were taken with a Leica DM 3000B microscope.

2.7 Western blot

To prepare protein samples, cells were collected with a cell scraper in PBS. After centrifugation, cell pellet was resuspended in IPH buffer (50mM Tris pH8, 150mM NaCl, 5mM EDTA 0.5% NP-40 v/v) containing 20X protease inhibitors (Roche) and 1X DTT (dithiothreitol, Thermo Fisher**)** and disrupted mechanically for 10 seconds before being incubated on ice for 30 minutes. Supernatant was collected after a 10 minute centrifugation at 1700 g and quantified using Coomassie Plus Bradford reagent (Thermo Fisher). Equal amounts of protein were mixed with 3X loading buffer (1M Tris-Cl pH 6.8, 3 ml of 20% SDS, 30% glycerol, 0.16% β-mercaptoethanol, bromophenol blue) heated at 95°C for 5 minutes before being cooled at room temperature, loaded onto a 10-12% sodium dodecyl sulfate polyacrylamide (SDS-page) gel and transferred to a PVDF membrane for 1.5 hours at 100 volts. Following transfer, membranes were blocked for 30 minutes in appropriate blocking buffer to minimize non-specific bands, containing 5% non-fat dry milk or 5% bovine serum albumin (BSA Sigma-Aldrich) in PBS-T. After blocking, membrane were incubated overnight at 4°C with primary antibody diluted in PBS-T with either 1% non-fat dry milk or 5% BSA, depending on the antibody. Membranes were washed 3 x 5 minutes with PBS-T before adding appropriate secondary antibody (anti-mouse or anti-rabbit). Detection of protein was done using an enhanced chemiluminescence reaction (ECL) and BioRad gel doc system (Bio-Rad, Hercules, CA, USA). Membranes could be re-probed with a different antibody by stripping with ReBlot Mild stripping solution (Millipore Billerica, Massachusetts, USA). Primary antibodies used are as follows: SMAD2/3 (Santa Cruz Biotechnology Dallas, Texas, USA and Cell Signalling Danvers, MA, USA), SMAD2 (Cell Signalling), pSMAD2/3 (Abcam), pSMAD2 (Cell Signalling), GKLF (Santa Cruz Biotechnology), C/EBPβ (Santa Cruz Biotechnology and Abcam), Myogenin (F5D, DSHB,

Iowa, USA), MYOD (Santa Cruz Biotechnology), MyHC (MF-20 from hybridoma), and cyclophilin-B (Abcam). Concentration of antibodies was used as directed by the manufacturer's data sheet (ranging from 1:500-1:1000 and 1:10 000 for cyclophilin-B). Secondary antibodies used are as follows: anti-mouse and anti-rabbit from GE Healthcare at a concentration of 1:5000.

2.8 RT qPCR

Cells were harvested in RLT buffer containing β-mercaptoethanol and RNA was extracted using the RNA Easy Mini Kit (Qiagen Hilden, Germany) according to the manufacturer's instructions. Quantification was performed using a Nanodrop (Thermo) and 1µg RNA was treated with Rnase-Free DNase Kit (Qiagen Hilden, Germany). First strand cDNA was made using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) using a standard thermocycler. Quantitative PCR was done using iTaq Universal SYBR Green Supermix on Bio-Rad thermocycler. CT values were analyzed with the delta delta CT method using 18S as an internal control (Livak and Schmittgen 2001). The primer sequences used were as follows:

Smad2 F: 5′-ATGTCGTCCATCTTGCCA-3′

Smad2 R: 5′-AACCGTCCTGTTTTCTTTAG-3′

Smad3 F: 5′-CGTAATTCATGGTGGCTGTG-3′

Smad3 R: 5′-ACCAAGTGCATTACCATCCC-3′

Cebpb F: 5′-TCGAACCCGCGGACTGCAAG-3′

Cebpb R: 5′-CGACGACGACGTGGACAGGC-3′

Pax7 F: 5′-GACGACGAGGAAGGAGACAA-3′

Pax7 R: 5′-CGGGTTCTGATTCCACATCT-3′

18s F: 5′-CGCCGCTAGAGGTGAAATC-3′

18s R: 5′-CCAGTCGGCATCGTTTATGG-3′

Myf5 F: 5′-GCTGAGGGAACAGGTGGAGA-3′

Myf5 R: 5′-CTGCTGTTCTTTCGGGACCAG-3′

Myod1 F: 5′-TGGCATGATGGATTACAGCG-3′

Myod1 R: 5′-CCACTATGCTGGACAGGCAGT-3′

Myogenin F: 5′-ATCGCGCTCCTCCTGGTTGA-3′

Myogenin R: 5′-CTGGGGACCCCTGAGCATTG-3′

neoMyHC F: 5′-TCGCTGGCTTTGAGATCTTT-3′

neoMyHC R: 5′-ACGAACATGTGGTGGTTGAA-3′

Klf4 F: 5′-GCAGTCACAAGTCCCCTCTC-3′

Klf4 R: 5′-TAGTCACAAGTGTGGGTGGC-3′

Npnt F: 5′-TGGAGGCAAACCCAGATCAC-3′

Npnt R: 5′- GCAGCGACCTCTTTTCAAGC-3′

2.9 Chromatin imunoprecipitation (ChIP)

For ChIP assay, cells were plated in 10cm plates (1 plate per condition and antibody) and cultured either in growth or differentiation media, as indicated in the figure legends. Briefly, at Day 1, cells were washed twice with serum free DMEM, trypsinized and incubated with 37% formaldehyde at room temperature, rotating for 10 minutes. Cells were centrifuged at 900 g for 4 min and cell pellet was resuspended in cold phosphate buffered saline (PBS). Cells were incubated for 10 minutes in each of the following buffers: Buffer I (0.25% Triton X-100, 10mM EDTA, 10mM HEPES pH6.5, 0.5mM EGTA), Buffer II (200mM NaCl, 1mM EDTA, 0.5mM EGTA, 10mM HEPES pH6.5). Cells were sonicated twice for 10sec each using Branson Sonicater 450 with a 2mm tip and 10% of power output in sonication buffer (1% SDS, 10mM EDTA, 50mM Tris pH 8.1, 1mM DTT, 1X protease inhibitors). For the immunoprecipitation,

cell supernatant was diluted in dilution buffer (1% Triton X-100, 150mM NaCl, 2mM EDTA, 20mM Tris pH 8.1, 1mM DTT, 1X protease inhibitors) with 2 µg of specific antibody, rotating overnight at 4ºC. Cell sample for input (25%) was diluted in Buffer E (1% SDS, 100mM $NaHCO_3$) and heated in a 65 ºC water bath overnight. At Day 2 of immunoprecipitation, 2 µg of sheared salmon sperm DNA and Protein G Dynabeads (Novex Life Technologies, Oslo, Norway) were added to cell samples and incubated at 4 ºC for 2 hours. Dynabeads were washed 10 minutes at 4 ºC once with the following buffers: TSE I (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH 8.1, 150mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20mM Tris pH 8.1, 500mM NaCl), TSE III (250mM LiCl, 1% NP-40, 1% Deoxycholate, 1mM EDTA, 10mM Tris pH 8.1) and 3 times with TE buffer (10 mM Tris-HCl pH 8 and 1 mM EDTA pH 8). Beads were extracted 3 times with Buffer E and heated at 65 ºC overnight. DNA purification was achieved with QIAquick PCR purification Kit (Qiagen Hilden, Germany) following the manufacturer's instructions and quantitative PCR was done using iTaq Universal SYBR Green Supermix on Bio-Rad thermocycler. CT values were analyzed with the delta delta CT method (Livak and Schmittgen 2001) using 10% input as an internal control. Antibodies used for ChIP were as follows: C/EBPβ (Santa Cruz Biotechnology), RAR (Santa Cruz Biotechnology), Smad2 (Cell Signalling), GKLF (Santa Cruz Biotechnology) and normal rabbit IgG (Invitrogen) as a control.

Primers used for RT-qPCR of ChIP samples were as follows:

Smad2 promoter F: 5′-AAGTCCCTGGAGGGAATGGA-3′

Smad2 promoter R: 5′-CACTGTAGGCAGAGCAGGTT-3′

Pax7 promoter F: 5′-CCCGAACTGGCCCCCTTTCC-3′

Pax7 promoter R: 5′-TCCCCCGGAGGACTGGAACG-3′

Intronic RARE Smad3 promoter F: 5′-ATGACTTGTTCCTGTCCTTC-3′

Intronic RARE Smad3 promoter R: 5′-GCTAGGCAGAGTTCCCAGAA-3′

Klf4 pro1 F: 5′-TATAACTTCTCGCTCGCTTGCTC-3′

Klf4 pro1 R: 5′-TGCGCGGAGTTTGTTTATTTAG-3′

Klf4 pro2 F: 5′- GAAAGTCCTGCCACGGGAA-3′

Klf4 pro2 R: 5′- CTGGATGAGTCACGCGGATAA-3′

Klf4 -10kb F: 5′- CAGGAATGCCTGTGGGGATAG-3′

Klf4 -10kb R: 5′- TGACCGGCTGAAGCTTTGTC-3′

Npnt pro1 F: 5′-GCTTTTCCTCTGGTCCCCTC-3′

Npnt pro1 R: 5′-GATGCCGCACCTGTTTTACC-3′

Npnt pro2 F: 5′-ATTGGCTTTCCTGTCCCTGG-3′

Npnt pro2 R: 5′-TTAGCCTCTGGCTGCTTTCC-3′

Npnt -12kb F: 5′- GGTTGGAACCGCAGTGAGTA-3′

Npnt -12kb R: 5′- GCAGTGACAACAGGGAGACA-3′

2.10 Immunocytochemistry for myosin heavy chain (MyHC) on cells

For immunocytochemistry, cells were washed twice with PBS and fixed with 100% methanol for 10 minutes. After 2 x 5 minutes washes with PBS, cells were permeabilized with 0.3% Triton-X in PBS for 15 minutes. After permeabilization, cells were incubated with myosin heavy chain (MF-20 from hybridomas) at a concentration of 1:50 in 0.3% Triton-X in PBS overnight at 4°C. Cy3 anti-mouse (Jackson ImmunoResearch, West Grove, PA, USA) was used as a secondary antibody and incubated for 1 hour, covered and at room temperature. DAPI (0.5 µg/ul Sigma-Aldrich) was used to reveal nuclei. Six pictures of a random field of view were taken using a microscope (Leica DM 3000B), Infinity-3 camera (Lumenera) and Infinity Capture

imaging software (Lumenera). Images were merged using the Paint.Net program (https://www.getpaint.net/index.html) and Image J (https://imagej.nih.gov/ij/) was used to merge and assess differentiation index (% cells that express MyHC/total), fusion index (#nuclei per myotube), % unfused nuclei and total cell numbers.

2.11 Hematoxylin and Eosin (H&E) staining on frozen sections

H & E staining on frozen muscle sections was done by first warming slides for 15 minutes at 37°C before fixing in 4% paraformaldehyde for 10 minutes. Slides were washed in PBS for 10 minutes and in water for 5 minutes before dipping in hematoxylin for 4 minutes. Slides were rinsed in water for 5 minutes before dipping in a 1% acid alcohol solution for 15 seconds and rinsed again in water for 1 minute. Counterstaining was done with Eosin for 30 seconds and subsequently incubated in 70% ethanol for 20 seconds, 100% ethanol twice for 2 minutes and finally in xylene twice for 5 minutes. Mounting was done with Permount and coverslips immediately after removal of xylene. Pictures were taken with a brightfield light microscope CX42 (Olympus, Richmond Hill, ON, Canada) using a Qcapture 3 camera (Surrey, BC, Canada).

2.12 Mammalian expression constructs

pLXSN and pLXSN-C/EBPβ retroviral expression vectors were previously described (Wiper-Bergeron et al. 2003). The MSCV CreERT2 puro retroviral vector was purchased from Addgene. The pLPCX, pLPCX-SMAD2 and pLPCX-SMAD2ΔSSMS plasmids were purchased from Addgene. The pGL3-*Klf4*-Luc promoter was kindly gifted by Dr. Christman at the Ohio State University (Karpurapu et al. 2014). The pMXs-KLF4 plasmid was a gift from Dr. Toshio Kitamura (Institute of Medical Science, The University of Tokyo, Japan) while the control

pMXs-Ctl plasmid was kindly gifted by Dr. William Stanford (Ottawa Hospital Research Institute, Ottawa, ON).

2.13 Dual Luciferase Reporter Assay

C2C12 cells were transiently transfected with an experimental promoter plasmid (1µg), mammalian constructs (1µg) and control Renilla plasmid (0.25µg), as indicated using FuGENE HD transfection reagent (Promega). Cells were supplemented with growth medium for 6 hours and collected 48 hours post-transfection, respectively. Using the Dual-Luciferase Reporter Assay Kit (Promega), cells were washed in PBS and lysed with 100 µl 1X Passive Lysis Buffer for 15 minutes, shaking at room temperature. Twenty µL of the whole cell extract sample was mixed with 100 µL Luciferase Assay Reagent (LAR) in a glass tube and read with a Monolight 2010 luminometer (Analytical Luminescence Laboratory Ann Arbor, MI, USA) to assess luciferase intensity. Signal was quenched using 100 µL Stop & Glo reagent to assess Renilla plasmid transfection efficiency. The ratio of Luciferase/Renilla was calculated and normalized to experimental control (promoter in absence of experimental plasmid).

2.14 Statistical analysis

Statistical analysis was conducted using the statistical analysis software GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA, https://www.graphpad.com/scientific-software/prism/). A Student's t-test was used when comparing two conditions. One-Way ANOVA was performed when comparing three or more treatments in one cell type. Two-way ANOVA was used when comparing two conditions in an experimental and control cell line. Post-hoc tests followed only statistically significant ANOVA results ($p < 0.05$). Where multiple comparisons are possible, means are marked with a letter code. Thus, means with different letters are significantly different from one another, meeting a minimum cutoff of $p < 0.05$. All experiments are representative of a

minimum of three biological replicates, as indicated. For all graphs, bars represent the mean and error bars represent the standard error mean (SEM); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

CHAPTER 3: RETINOIC ACID ANTAGONIZES TGFβ SIGNALLING BY UPREGULATING SMAD3

3.1 Retinoic acid upregulates *Smad3* expression in myoblasts

Our lab has previously observed that treatment of adipocytes with RA upregulates SMAD3 expression, without affecting the expression of SMAD2 (Marchildon, 2010). The RA-induced increase in SMAD3 expression led to the displacement of C/EBPβ from its response element in the *Cebpa* promoter, a master regulator of adipogenesis, thereby inhibiting this differentiation process (Marchildon et al. 2010; Wiper-Bergeron, St-Louis, and Lee 2007). Since C/EBPβ is expressed in primary and C2C12 myoblasts, we asked whether RA could also upregulate SMAD3 in myoblasts and thereby disrupt C/EBPβ transcriptional activity in this system (Marchildon et al. 2012). C2C12 myoblasts were treated with 1nM RA in both growth (GM) and low-serum conditions (DM) for 48 hours, a time point previously determined to upregulate SMAD3 in adipocytes (Marchildon et al. 2010). Quantitative reverse transcriptase-PCR analysis (RT-qPCR) revealed that RA treatment upregulates *Smad3* mRNA expression in C2C12 cells over 2-fold in both growth and low-serum conditions as compared to vehicle treatment (Figure 5A, B). To ensure RA treatment was not toxic to the cells during prolonged exposure, such as under differentiation conditions, different concentrations of RA (0, 1, 10, 100 nM) were tested on C2C12 myoblasts during differentiation for 96 hours and total nuclei were counted (Figure 5C-D). While cell numbers were not affected by a 1 nM RA treatment as compared to vehicle treatment, concentrations of 10 nM and 100 nM significantly reduced the total nuclei in the field of view from 800 to 400 nuclei (Figure 5D). Furthermore, a growth curve assay, demonstrated that C2C12 myoblasts cells treated with 1 nM RA grew comparably to vehicle-treated cells (Figure 5E). While RA upregulated *Smad3* expression, and this effect was shown to be due to *de novo* transcription in pre-adipocytes (Marchildon et al. 2010), analysis of the *Smad3* regulatory region (-5 kb) failed to reveal retinoic acid response elements (RARE); binding sites for RA-

activated retinoic acid receptor-retinoid X receptor (RAR-RXR) heterodimers. However, a RARE motif was found between Exon 3 and Exon 4 of the *Smad3* gene (Figure 5F). Chromatin immunoprecipitation (ChIP) analysis of RAR occupancy of the intronic *Smad3* RARE revealed that RAR occupied this intronic binding site in C2C12 myoblasts (Figure 5G). These results suggest that RA upregulates *Smad3* in myoblasts and that this regulation occurs, in part, through the presence of RAR binding to RARE sites within *Smad3.*

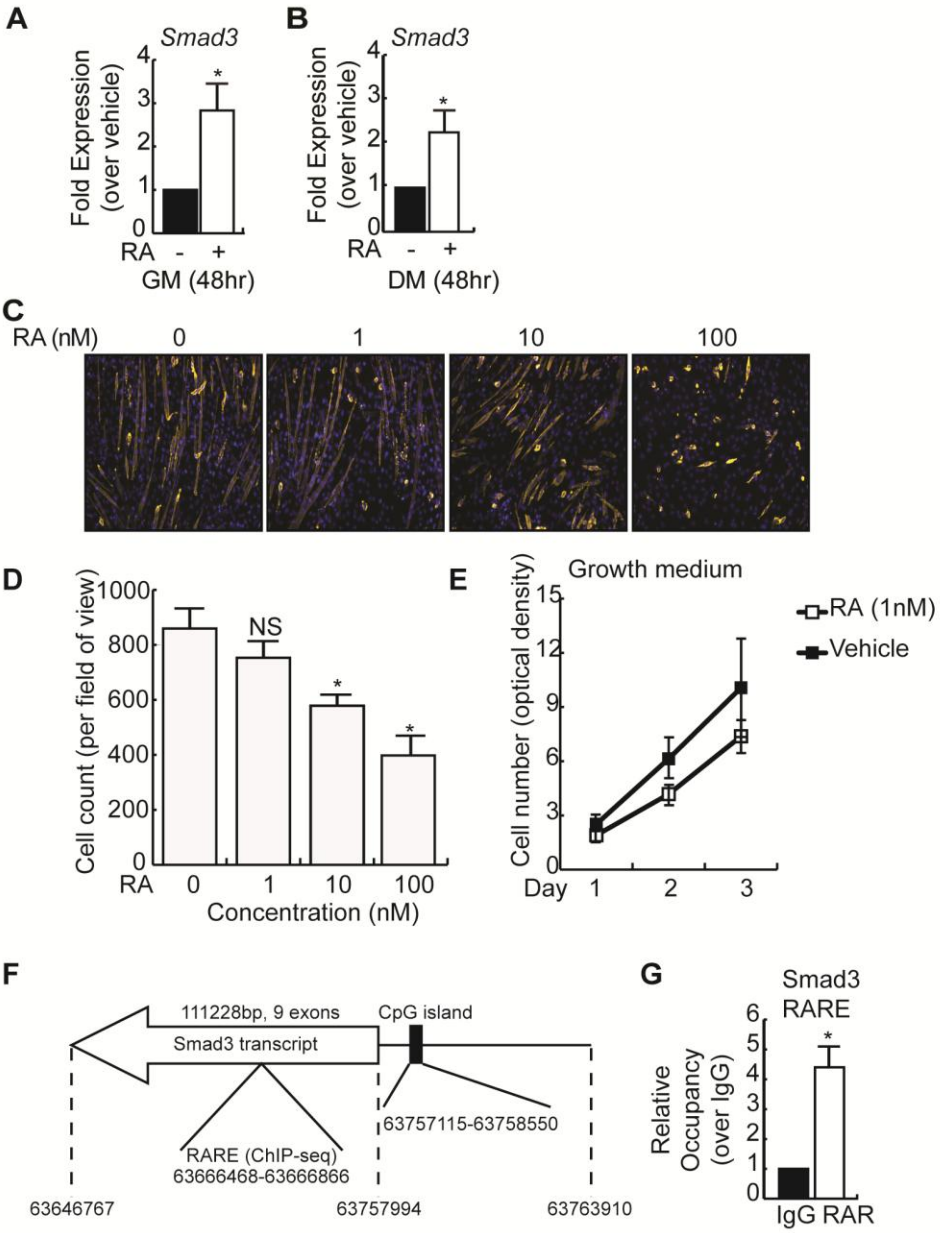

Figure 5. Retinoic acid upregulates Smad3 expression in myoblasts. **(A)** RT-qPCR analysis of *Smad3* mRNA expression in C2C12 myoblasts cultured in growth medium (GM) and treated with vehicle or RA for 48 h. Data is shown as fold expression over vehicle-treated condition; $*P < 0.05$, $n = 3$. **(B)** RT-qPCR analysis of *Smad3* mRNA expression in C2C12 myoblasts cultured in low-serum conditions (DM) and treated with vehicle or RA for 48 h. Data is shown as fold expression over vehicle-treated condition. $*P < 0.05$, $n = 3$. **(C)** C2C12 myoblasts were induced to differentiate in low serum conditions for 96 h, with vehicle or RA at indicated doses, after which immunocytochemistry for myosin heavy chain was performed. Representative images are shown; DAPI was used to reveal the nuclei. **(D)** DAPI cell counts per field of view at increasing doses of RA in C2C12 cells differentiated as in (C) for 96 h. $*P < 0.05$, NS = not significant, $n = 5$. **(E)** Crystal violet assay was performed to quantify C2C12 cell numbers (measured as dye intensity) after 1, 2, or 3 days in growth medium with vehicle or 1 nM RA; $n = 3$. **(F)** Schematic representation of the mouse *Smad3* locus found on the minus strand of chromosome 9 using the Mouse Dec. 2011 (GRCm38/mm10) Assembly. Location of the transcript, including 5′ and 3′ UTRs, is indicated as well as the predicted CpG island. The position of a retinoic acid response element (RARE) identified by ChIP-seq analysis in embryonic stem cells undergoing neurogenesis (GSM482750) between exon 3 and 4 of the *Smad3* gene is also indicated. **(G)** Analysis of retinoic acid receptor (RAR) occupancy of the RARE in the intronic region of the mouse *Smad3* gene by ChIP and RT-qPCR in C2C12 cells in growth medium. For all graphs, data represents the mean and error bars represent the SEM; $*P < 0.05$, minimum of 3 biological replicates used, as indicated.

3.2 Retinoic acid partially rescues the inhibition of myogenic differentiation induced by TGFβ

Previous studies showed that RA, at a low concentration, could promote myogenesis in P19 embryonic cells and was able to reverse the anti-myogenic effects of TGFβ (Edwards and McBurney 1983; Alric et al. 1998; Ryan et al. 2012; Kennedy et al. 2009; Hamade et al. 2006; Krueger and Hoffmann 2010). To further investigate the blockade of myogenic differentiation by TGFβ and the mechanism of action of RA on TGFβ-treated myoblasts, C2C12 myoblasts were induced to differentiate in low-serum conditions for 4 days, a time-point used to assess terminal differentiation and fusion, in the presence of vehicle, RA, TGFβ or co-treatment with RA and TGFβ (Figure 6A). TGFβ treatment potently inhibited both the differentiation and fusion of myoblasts, as shown by an important decrease in the differentiation index (# of MyHC+ cells/ total nuclei) and fusion index (# myonuclei per myotube) which were decreased 90% and 63%, respectively, as compared to control myoblasts (Figure 6B, C). Visualization of the contractile protein MyHC (yellow) by immuncytochemistry showed more myotubes and differentiated myocytes following treatment with RA which was confirmed by a higher differentiation index (Figure 6A, B). Co-treatment of myoblasts with RA and TGFβ led to a partial rescue of the differentiation index, and a full rescue of the fusion index, as compared to vehicle-treated cells (Figure 6B, C). The differentiation index of myoblasts co-treated with RA and TGFβ was restored to 55% of control cells (Figure 6B).

To further assess the molecular profiles of myoblasts treated with RA and TGFβ during differentiation, RT-qPCR analysis was performed for *Smad2*, and *Smad3*, the undifferentiated state marker *Pax7*, and the myogenic regulatory factors *Myf5*, *Myod1* and *Myog* (Figure 6D). RT-qPCR analysis revealed an increase in *Smad3* mRNA expression with RA treatment (white

bars), but not *Smad2* levels, after 4 days in low-serum conditions as compared to controls (Figure 6D). *Myog* levels were also increased by RA treatment, consistent with robust differentiation (Figure 6D). This increased differentiation observed in RA-treated myoblasts was independent of changes in *Myod1* and *Myf5* mRNA expression. Furthermore, RA treatment did not affect *Pax7* levels as compared to vehicle control (Figure 6D). Treatment with TGFβ (blue bars) increased both *Pax7* and *Myf5* expression, while inhibiting *Myod1* and *Myog,* a profile consistent with committed, undifferentiated myoblasts and decreased differentiation (Figure 6D). At this time-point, levels of *Smad2* and *Smad3* were not affected by TGFβ treatment compared to controls (Figure 6D). Addition of both RA and TGFβ (orange bars) during myoblast differentiation led to a decrease in *Pax7* levels compared to TGFβ-treated cells alone, consistent with improved differentiation compared to TGFβ treatment alone. In these conditions, *Myf5* and *Myod1* levels were unchanged. However, co-treatment resulted in a complete rescue of *Myog* levels, which was restored to vehicle-treated cells, consistent with an ameliorated differentiation profile (Figure 6A, B, C). Taken together, our results suggest that RA treatment can partially reverse the inhibitory effects of TGFβ during myogenic differentiation, in part by restoring *Myog* expression to control levels and decreasing *Pax7* compared to TGFβ-treated myoblasts.

We have previously shown in pre-adipocytes and osteoblasts, that the inhibition of C/EBPβ occupancy of target genes was achieved through RA-mediated SMAD3 upregulation, and was independent of classical C-terminal serines phosphorylation (Marchildon et al. 2010; Dingwall et al. 2011). As classical TGFβ signalling functions through C-terminal phosphorylation (serines 423 and 425) of the effector protein SMAD3 leading to nuclear translocation, we sought to determine whether the increase in *Smad3* expression following RA treatment observed in Figure 5A and Figure 5B also caused an increase in C-terminal phosphorylation of SMAD3. To this

end, we performed western analysis of C2C12 myoblasts treated with RA, TGFβ, or co-treatment with RA and TGFβ for 24 hours (Figure 6E). The antibody used in this experiment detects phosphorylation of the two TGFβ receptor-targeted C-terminal serines in both SMAD2 (higher band) and SMAD3 (lower band). C-terminal phosphorylation of SMAD2 or SMAD3 was not observed in untreated cells, however, SMAD2 and SMAD3 expression was detected (Figure 6E). Treatment with RA did not change SMAD2 or SMAD3 C-terminal phosphorylation status, while TGFβ treatment markedly induced phosphorylation of both SMAD2 and SMAD3 (Figure 6E). These results suggest that RA increases SMAD3 expression without affecting C-terminal SMAD3 phosphorylation, a hallmark of classical TGFβ signalling. Interestingly, co-treatment with RA and TGFβ reduced phospho-SMAD2 levels without affecting phospho-SMAD3 or SMAD2 protein expression, suggesting that RA may interfere with the actions of TGFβ by reducing SMAD2 activation by phosphorylation (Figure 6E).

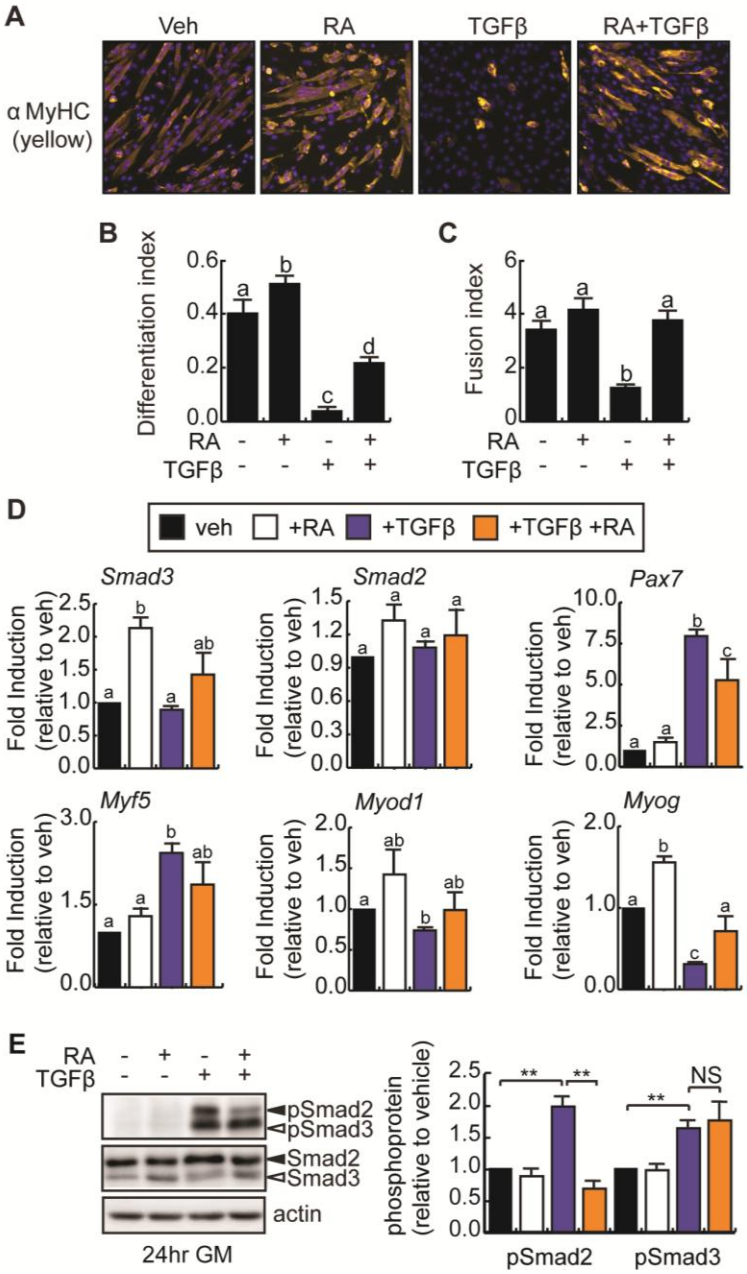

Figure 6. Retinoic acid partially rescues the inhibition of myogenesis induced by TGFβ. **(A)** Representative images of C2C12 myoblasts differentiated in low serum conditions in the continual presence of veh, RA, TGFβ or co-treatment with RA and TGFβ, as indicated, for 96 h and immunostained for myosin heavy chain (MyHC) expression. DAPI is used to reveal nuclei. **(B)** Differentiation index (#MyHC+nuclei/#total nuclei) of C2C12 cells cultured and treated as in (A). Means with different letters are significantly different from one another with a $P < 0.05$, $n = 3$. **(C)** Fusion index (#myonuclei/#myotubes) of C2C12 cells cultured and treated as in (A). Calculations include mononucleated myosin heavy chain positive cells. Means with different letters are significantly different from one another with a $P < 0.05$, $n = 3$. **(D)** RT-qPCR analysis of *Smad3*, *Smad2*, *Pax7*, *Myf5*, *Myod1* and *Myog* expression in C2C12 myoblasts treated and differentiated as in (A). Means with different letters are significantly different from one another with a $P < 0.05$, $n = 4$. **(E)** Western analysis of phosphorylated SMAD2 and SMAD3 (pSMAD2/3) expression in C2C12 cells in growth conditions. Quantification of western blots is represented as pSMAD2 or pSMAD3 relative to actin, respectively, $n = 3$, $^{**}P < 0.01$, NS = not significant. Protein levels for SMAD2 and SMAD3 are shown. For all graphs, data represents the mean and the error bars represent the SEM.

3.3 TGFβ treatment stimulates C/EBPβ expression in myoblasts

TGFβ potently inhibits myogenic differentiation and our own studies show that C/EBPβ is a negative regulator of differentiation (Marchildon et al. 2012). Additionally, given that TGFβ treatment increased *Pax7* mRNA levels 8-fold in myoblasts (Figure 6D), and that *Pax7* is a C/EBPβ target gene (Marchildon et al. 2012), we sought to evaluate C/EBPβ expression in both TGFβ and RA-treated myoblasts. C2C12 myoblasts were treated with TGFβ and induced to differentiate for 4 days in low-serum conditions (DM) after which levels of C/EBPβ and PAX7 were assessed by western blot (Figure 7). Western analysis and subsequent quantification revealed a robust increase in the protein expression of both C/EBPβ and PAX7 with addition of TGFβ, compared to vehicle treated cells (Figure 7A; quantified in B and C). Furthermore, while RA treatment alone did not affect C/EBPβ expression; in the presence of TGFβ, addition of RA did reduce C/EBPβ expression as compared to TGFβ treatment alone (Figure 7D; quantified in E). RT-qPCR analysis also revealed an increase in *Cebpb* levels with TGFβ treatment, in accordance with protein expression, while RA treatment alone did not have an effect compared to vehicle-treated cells (Figure 7F). Moreover, co-treatment of RA and TGFβ reduced *Cebpb* mRNA back to control levels (Figure 7F). These results suggest that C/EBPβ protein and *Cebpb* mRNA expression is regulated by TGFβ in C2C12 myoblasts and that RA can antagonize the TGFβ-induced increase of C/EBPβ in this context.

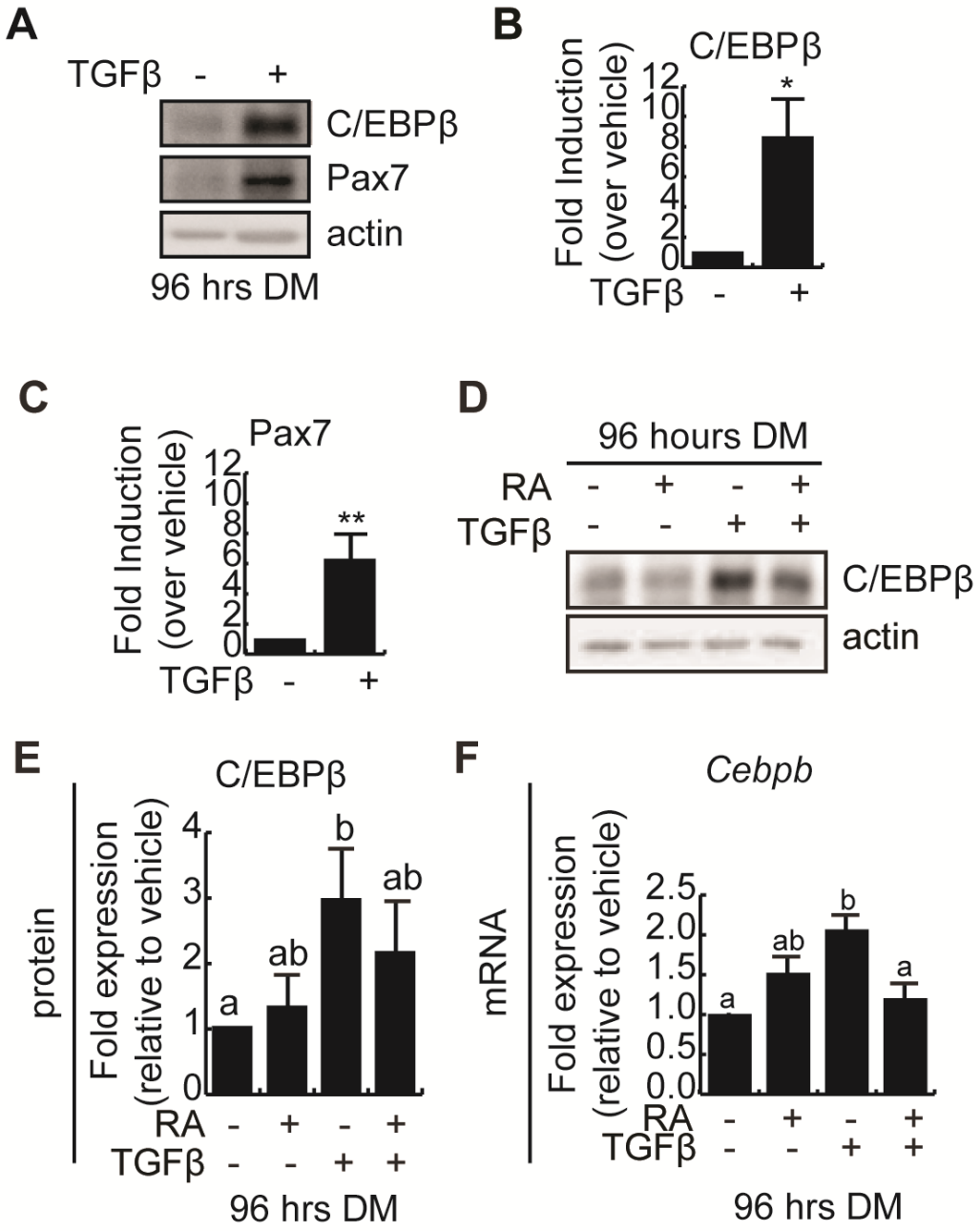

Figure 7. TGFβ treatment stimulates C/EBPβ expression in myoblasts. (A) Western analysis of C/EBPβ and PAX7 expression in C2C12 myoblasts cultured in differentiation medium in the presence or absence of TGFβ for 96 h. Actin is used as a loading control. **(B)** Quantification of C/EBPβ expression from western blots in panel (A) relative to vehicle-treated control. Error bars are the SEM, $n = 3$, $^{*}P < 0.05$. **(C)** Quantification of PAX7 expression from western blots in (A) relative to vehicle-treated controls. Error bars are the SEM, $n = 3$, $^{**}P < 0.01$. **(D)** C/EBPβ protein expression after 96 h in differentiation medium (DM) in the continual presence of veh, RA, TGFβ or co-treatment with RA and TGFβ, as indicated. Actin is used as a loading control. **(E)** Quantification of western blots from (D) relative to the vehicle-treated controls; $n = 4$. **(F)** RT-qPCR analysis of *Cebpb* expression in C2C12 myoblasts induced to differentiate with veh, RA, TGFβ or co-treatment with RA and TGFβ, as indicated, for 96 h. Data is shown relative to vehicle-treated controls; $n = 3$. For all graphs data represents the mean and error bars are the SEM. Means marked by different letters are statistically different from one another, meeting the minimum cutoff of $P < 0.05$.

3.4 Retinoic acid interferes with C/EBPβ occupancy of target genes

Previous results from our lab demonstrate a role for RA-induced SMAD3 in disrupting C/EBPβ transcriptional activities on its target promoters *Cebpa* and *Runx2*, during adipogenesis and osteoblastogenesis, respectively (Marchildon et al. 2010; Dingwall et al. 2011; Wiper-Bergeron, St-Louis, and Lee 2007). We have also demonstrated that *Pax7* is a C/EBPβ target during myogenesis (Marchildon et al. 2012). To determine if RA could disrupt the interaction of C/EBPβ with the *Pax7* promoter in myoblasts, we performed a chromatin immunoprecipitation (ChIP) experiment to measure C/EBPβ occupancy of the *Pax7* promoter in growth medium (GM). This time point was chosen since PAX7 levels are high in GM, and decrease with onset of differentiation (Figure 8). While C/EBPβ occupies the *Pax7* promoter in vehicle-treated cells, treatment with RA decreased the occupancy of C/EBPβ on the *Pax7* promoter in GM (Figure 8A). Further, RA treatment completely blocked C/EBPβ occupancy of the *Pax7* promoter in myoblasts induced to differentiate in low-serum conditions (DM); decreasing relative C/EBPβ occupancy from a 3-fold increase in vehicle conditions back to control levels in the presence of RA (Figure 8B). Next, RT-qPCR analysis was performed to correlate changes in occupancy with changes in *Pax7* levels. Under growth conditions, *Pax7* levels were unaffected by treatment with RA (Figure 8C). However, in low-serum conditions we observed a 40% decrease in *Pax7* expression when compared to vehicle treatment, consistent with the C/EBPβ ChIP experiment (Figure 8D).

Previous results from our lab indicate that C/EBPβ levels are high in proliferating myoblasts, decrease with differentiation and if C/EBPβ expression is forced, myogenic differentiation is blocked, concomitant with increased PAX7 and decreased MYOD, myogenin and MyHC expression (Marchildon et al. 2012). However, the mechanism by which this control

of myogenesis is achieved remains unknown. Given that TGFβ treatment and overexpression of C/EBPβ both result in the inhibition of myogenic differentiation with a similar molecular profile, we sought to determine whether the expression of SMAD2 and SMAD3 was regulated by C/EBPβ and had a role a role in this context . Therefore, we retrovirally transduced C2C12 cells to express C/EBPβ or an empty vector control (pLXSN) and cultured the cells in growth conditions in the presence or absence of RA. As seen previously, a 48 hour RA treatment increased SMAD3 expression in both control and C/EBPβ overexpressing cells, compared to vehicle-treated control (Figure 8E; quantified in F). Consistent with previous findings in pre-adipocytes and mesenchymal stem cells, RA treatment did not impact C/EBPβ expression in empty vector control cells or in cells overexpressing C/EBPβ (Figure 8E, F). Overexpression of C/EBPβ alone caused an increase in SMAD2, but not SMAD3 expression, which RA treatment was able to completely reverse back to control levels (Figure 8E, F). These results suggest that SMAD2 is a potential C/EBPβ target in myoblasts and that RA can antagonize its upregulation.

To further address the regulation of SMAD2 by C/EBPβ, primary myoblasts were taken from C/EBPβ conditional knockout mice (*Cebpb* cKO), where *Cebpb* is excised in PAX7-expressing satellite cells upon tamoxifen treatment, and *Smad2* levels were analyzed. RT-qPCR analysis revealed a 60% decrease in *Smad2* mRNA levels in *Cebpb* cKO myoblasts in growth conditions (GM) compared to controls (Figure 8G, H). Given that i) C/EBPβ regulates *Smad2* expression and that ii) RA-induced SMAD3 could disrupt C/EBPβ transcriptional activity, we asked whether C/EBPβ occupied the *Smad2* promoter and if RA could disrupt C/EBPβ occupancy of this promoter. Indeed, C/EBPβ was found to occupy the *Smad2* promoter region in growth conditions and addition of RA completely abolished this occupancy (Figure 8I). Moreover, RA treatment decreased *Smad2* mRNA levels by 30% and 40% as compared to

controls in growth and differentiation conditions, respectively (Figure 8J, K). A 24-hour RA treatment also decreased *Cebpb* levels in low-serum conditions, and while trending towards a decrease in growth conditions, this did not meet statistical significance (Figure 8L, M). These results suggest that RA can interfere with C/EBPβ occupancy of the *Pax7* and *Smad2* promoters, leading to a decrease in their gene expression in myoblasts. Therefore, RA-induced SMAD3 interferes with C/EBPβ transcriptional activities, which decreases *Smad2* expression and would thereby reduce TGFβ-mediated signalling.

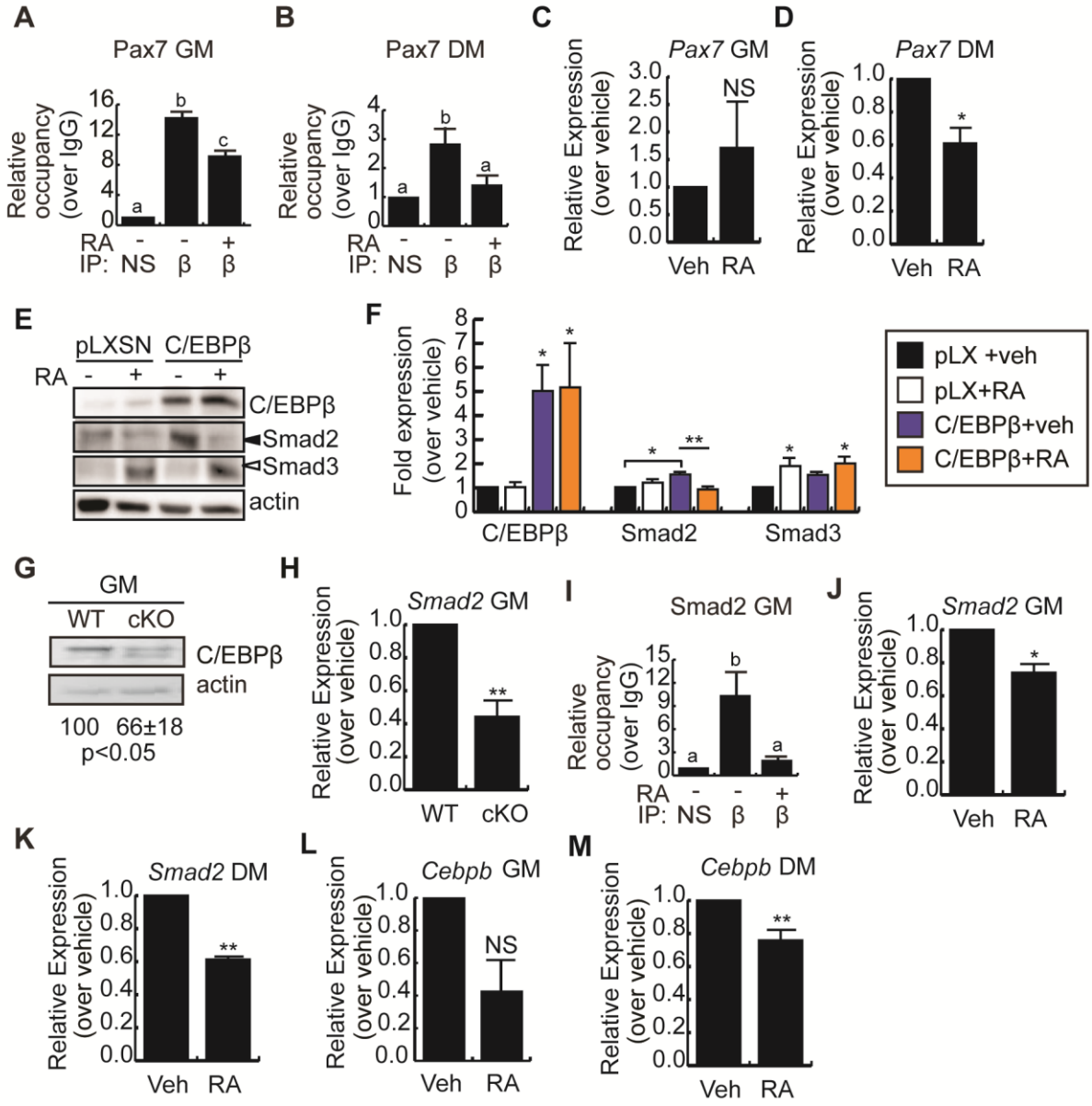

Figure 8. Retinoic acid interferes with C/EBPβ occupancy of target genes. **(A)** C/EBPβ occupancy of the *Pax7* promoter in C2C12 myoblasts in GM -/+RA for 24h; $n = 3$. **(B)** C/EBPβ occupancy of the *Pax7* promoter in C2C12 myoblasts in DM -/+RA for 24h; $n = 3$. **(C)** RT-qPCR analysis of *Pax7* expression in C2C12 myoblasts -/+RA treatment for 24h in GM; $n = 3$. **(D)** *Pax7* mRNA expression in C2C12 myoblasts after 24 h -/+RA in DM; $n = 3$, $^{*}P < 0.05$. **(E)** SMAD2, SMAD3 and C/EBPβ expression in C2C12 myoblasts retrovirally transduced to express C/EBPβ or with empty virus (pLXSN) -/+ RA for 48h in GM. Actin is the loading control. **(F)** Quantification of western blots from (E); $^{*}P < 0.05$, compared to vehicle-treated empty virus controls; $^{**}P < 0.01$, compared to C/EBPβ-overexpressing vehicle-treated cells, $n \geq 3$. **(G)** C/EBPβ expression in primary myoblasts from C/EBPβ conditional null (cKO) or control (WT) muscle in GM. Quantification of 3 trials is indicated, with control set to 100, $P < 0.05$. **(H)** *Smad2* mRNA expression in control (WT) and cKO myoblasts in GM, $n = 3$. **(I)** ChIP analysis of C/EBPβ occupancy of the *Smad2* promoter in C2C12 myoblasts after 24h -/+RA in GM, $n = 3$. **(J)** *Smad2* expression in C2C12 myoblasts treated for 24h -/+RA in GM, $n = 3$. **(K)** *Smad2* expression in C2C12 myoblasts treated for 24h -/+RA in DM, $n = 3$. **(L)** *Cebpb* expression in cells cultured as in (J). **(M)** *Cebpb* expression in cells cultured as in (K), $n = 3$. For all graphs, data is the mean and error bars represent the SEM. Means marked with different letters are statistically different from one another, meeting a minimum cutoff of $P < 0.05$. $^{*}P < 0.05$, $^{**}P < 0.01$, NS = non significant.

3.5 Inhibition of myogenic differentiation by TGFβ is partially rescued by loss of C/EBPβ expression

Given that TGFβ upregulates C/EBPβ expression in myoblasts, we wanted to evaluate whether loss of C/EBPβ would rescue the TGFβ-induced inhibition of myogenic differentiation. To this end, primary myoblasts were isolated from C/EBPβ conditional null mice (cKO: *Cebpb*$^{fl/fl}$/*Pax7*$^{CreER/+}$) or littermate non-Cre-expressing controls (WT: *Cebpb*$^{fl/fl}$/*Pax7*$^{+/+}$) and cultured in growth conditions with 4-OH tamoxifen for 2 days. WT and *Cebpb* cKO myoblasts were induced to differentiate for 2 days in low-serum conditions in the presence or absence of TGFβ (Figure 9A). Immunocytochemistry for the contractile protein MyHC and quantification of the differentiation index (#MyHC +nuclei/total nuclei) and fusion index (#nuclei/myotube) revealed no difference between WT and *Cebpb* cKO myoblasts in the absence of TGFβ treatment (Figure 9A, B, C). However, we previously demonstrated that *Cebpb* cKO myotubes were larger (increased fusion index), and believe this discrepancy is a result of the imperfect excision rate and low cell numbers obtained in this experiment. As expected, TGFβ treatment inhibited differentiation in WT cells as seen by a 67% decrease in the differentiation index as compared to vehicle-treated cells (Figure 9B). In *Cebpb* cKO myoblasts, TGFβ treatment reduced the differentiation index by approximately 38% as compared to vehicle-treated WT cells, which when considered in the context of the imperfect excision of *Cebpb* in these cells, suggests that C/EBPβ is required for the inhibition of myogenesis by TGFβ (Figure 9B). TGFβ treatment significantly reduced myotube size, resulting in a 50% decrease in the fusion index in WT myoblasts (Figure 9C). TGFβ-treated *Cebpb* cKO myoblasts trended towards an increased fusion index compared to TGFβ-treated WT myoblasts; however this increase did not reach statistical

significance (Figure 9C). These results indicate that loss of C/EBPβ can partially rescue the TGFβ-induced inhibition of myogenic differentiation.

To evaluate the molecular profile of differentiating WT and *Cebpb* cKO cells in the presence or absence of TGFβ, RT-qPCR analysis was performed for *Smad2*, *Smad3*, *Cebpb*, *Pax7*, *Myhc* and MRF (*Myod1*, *Myf5* and *Myog*) expression. Excision of *Cebpb* in the cKO myoblasts was confirmed by a 45% reduction in this mRNA compared to WT (Figure 9D). TGFβ did not increase *Cebpb* levels in WT primary myoblasts, as it did in C2C12 cells. As observed in the previous figure, *Smad2*, but not *Smad3* levels, were decreased in *Cebpb* cKO cells compared to WT in the absence of TGFβ (Figure 9D). *Smad3* was decreased by TGFβ treatment in both genotypes, while *Smad2* was decreased only in *Cebpb* cKO cells in this condition. *Pax7* levels did not change in *Cebpb* cKO cells compared to WT in either conditions, but were increased in TGFβ-treated WT cells, similar to C2C12 myoblasts. While *Myf5* and *Myod1* levels were unchanged in *Cebpb* cKO compared to WT cells, TGFβ treatment decreased the expression of these factors in both genotypes (Figure 9D). *Myog* levels remained unchanged in all 3 experimental conditions compared to vehicle-treated WT myoblasts (Figure 9D). Despite *Myhc* levels being decreased in both TGFβ-treated WT and *Cebpb* cKO cells compared to vehicle-treated WT controls, we observed a partial rescue of MyHC expression by immunocytochemistry in TGFβ-treated *Cebpb* cKO myoblasts (Figure 9A, D). Consistent with mRNA levels, western analysis showed a decrease in C/EBPβ expression in cKO cells which was also decreased in the presence of TGFβ (Figure 9E, F). SMAD2 protein expression was decreased in TGFβ-treated *Cebpb* cKO myoblasts only, while SMAD3 expression was decreased in WT and *Cebpb* cKO cells in the presence of TGFβ (Figure 9E). Further, myogenin expression was fully rescued to control levels in TGFβ-treated *Cebpb* cKO myoblasts (Figure 9E, F). MyHC

expression, although trending towards an increase in TGFβ-treated *Cebpb* cKO myoblasts, failed to reach statistical significance. Taken together, these results suggest that cells with lower levels of C/EBPβ are less sensitive to inhibition of differentiation by TGFβ. Thus, TGFβ inhibits differentiation at least in part through the stimulation of C/EBPβ, and by promoting C/EBPβ action through suppression of SMAD3 expression.

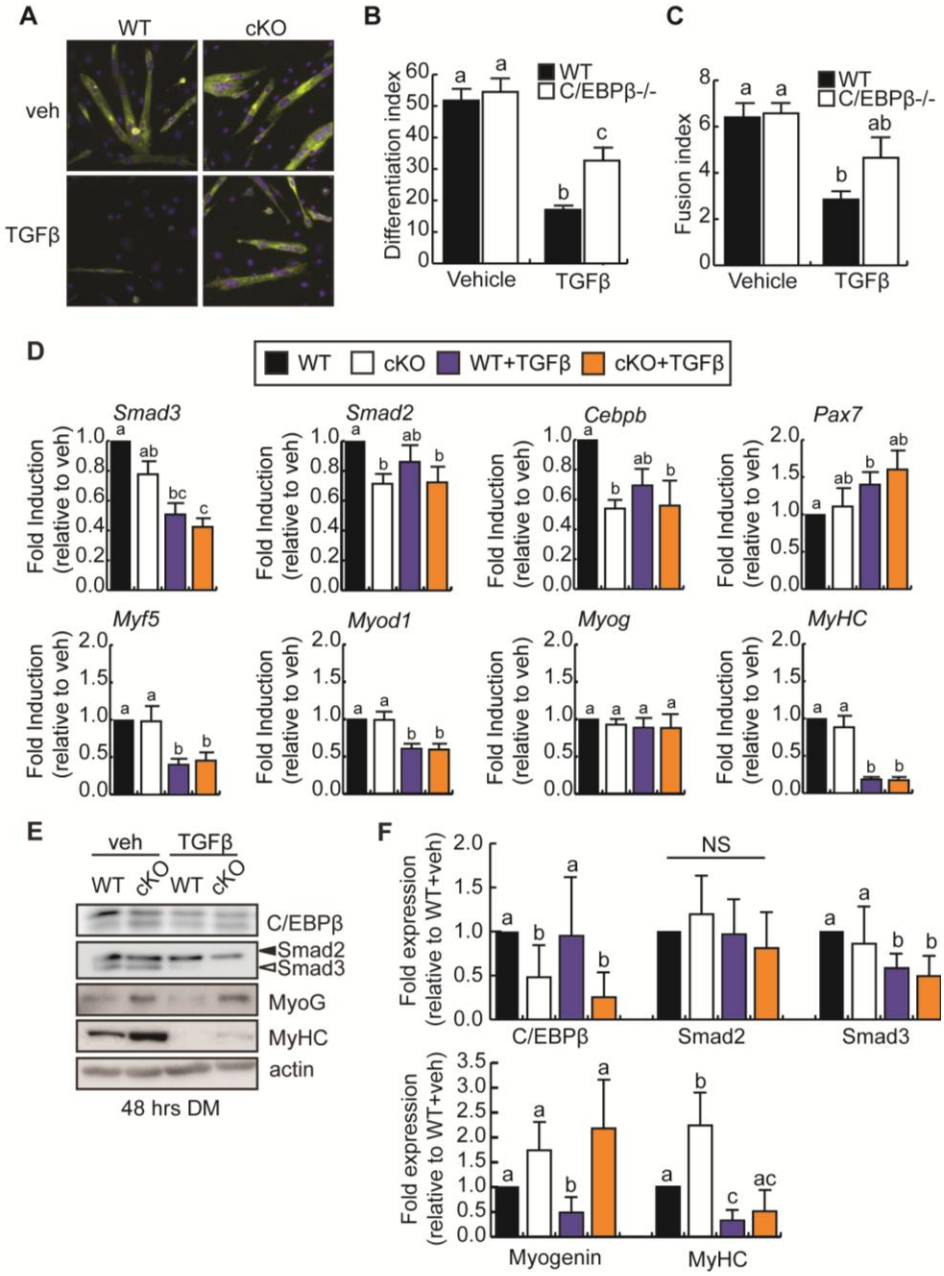

Figure 9. Inhibition of myogenic differentiation by TGFβ is partially rescued by loss of C/EBPβ expression. **(A)** Representative images of myosin heavy chain expression by immunocytochemistry in primary myoblasts isolated from cKO or control (WT) mouse hindlimb and induced to differentiate in low serum for 48h with vehicle or TGFβ treatment. **(B)** Differentiation index (#MyHC+nuclei/#total nuclei) of cells cultured and treated as in (A). Means marked by different letters are statistically different from one another, with a minimum of $P < 0.05$, $n = 3$. **(C)** Fusion index (#myonuclei/#myotubes) of cells cultured and treated as in (A). Counts exclude mononucleated myosin heavy chain positive cells. Means marked by different letters are statistically different from one another, meeting a minimum cutoff of $P < 0.05$, $n = 3$. **(D)** RT-qPCR analysis of *Smad2*, *Smad3*, *Cebpb*, *Pax7*, *Myf5*, *Myod1*, *Myog* and *MyHC* in primary myoblasts differentiated as in (A). Means with different letters are significantly different from one another with a $P < 0.05$, $n \geq 3$. **(E)** Representative western blots of C/EBPβ, SMAD2, SMAD3, MYOG, and myosin heavy chain in primary myoblasts differentiated as in (A). Means with different letters are significantly different from one another with a $P < 0.05$, $n = 5$. **(F)** Quantification of C/EBPβ, SMAD2, SMAD3, myogenin, and myosin heavy chain protein expression from (E) relative to vehicle-treated WT control cells. Means marked with different letters are statistically different from one another, meeting a minimum cutoff of $P < 0.05$, $n \geq 3$. For all graphs data represents the mean and error bars are the SEM.

CHAPTER 4: CHARACTERIZATION OF SATELLITE CELL-SPECIFIC SMAD2 CONDITIONAL KNOCKOUT MICE

Thus far, we have identified a pro-myogenic role for RA-induced SMAD3 expression, which remains unphosphorylated at its C-terminus but can abrogate C/EBPβ occupancy of target genes *Pax7* and *Smad2* during myogenesis (summarized in Figure 10). Although TGFβ-activated SMAD3 is thought to be a negative regulator of myogenic differentiation, little is known about the specific role of SMAD2 in this process. Indeed we have observed higher levels of SMAD2, compared to SMAD3 expression, in both C2C12 and primary myoblasts, and sought to investigate its role in myogenic differentiation. Given that i) TGFβ upregulated C/EBPβ expression and forced expression of C/EBPβ increased SMAD2 expression; ii) TGFβ treatment and C/EBPβ overexpression inhibit myogenic differentiation, and iii) RA treatment decreased TGFβ-induced C/EBPβ and C/EBPβ-induced SMAD2 levels; we hypothesized an anti-myogenic role for SMAD2 as the main effector of TGFβ action in myoblasts. We investigated the role of SMAD2 specifically in myoblasts using gain of function experiments and by creating a novel murine model where *Smad2* is excised in PAX7-expressing satellite cells.

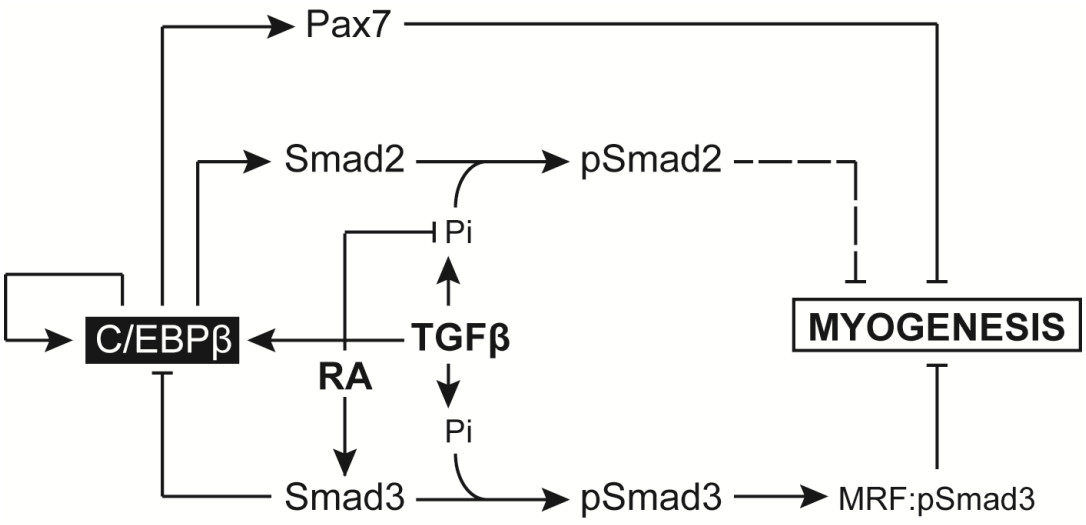

Figure 10. Proposed pro-myogenic role for retinoic acid-induced SMAD3. The bZIP transcription factor C/EBPβ is a potent inhibitor of myogenesis. It can stimulate the expression of PAX7 and SMAD2 and can autoregulate its own expression. Regulation of PAX7 expression suppresses myogenic differentiation, while the contribution of SMAD2 to this process remains unknown. TGFβ treatment can stimulate C/EBPβ expression during myogenic differentiation and this contributes in part to the anti-myogenic effects of TGFβ, as loss of C/EBPβ partially restores differentiation in the presence of TGFβ. TGFβ can also, upon binding its receptor, stimulate the phosphorylation of both SMAD2 and SMAD3 and it is known that SMAD3 can form inhibitory complexes with myogenic regulatory factors and inhibit myogenesis. Treatment with retinoic acid (RA) antagonizes the effects of TGFβ during myogenic differentiation. In particular, RA stimulates the expression of SMAD3 which in turn associated with C/EBPβ and reduces its occupancy of target gene promoters, resulting in a reduction of their expression and the restoration of differentiation. RA treatment also abrogates C-terminal phosphorylation of SMAD2 TGFβ, without affecting SMAD3 phosphorylation.

4.1 SMAD2 is regulated during myogenic differentiation

To begin investigating the role of SMAD2 during myogenesis, we sought to determine the expression pattern of *Smad2* mRNA during myoblast differentiation. Primary myoblasts were freshly isolated from C57BL/6 mice and a time course was performed by RT-qPCR analysis in growth conditions (GM) and 6h, 12h, 24h, 48h and 72h after induction to differentiate in low-serum conditions (Figure 11A). RT-qPCR analysis revealed that while *Smad2* was expressed in primary myoblasts, *Smad2* mRNA levels did not significantly change during the differentiation time course, compared to GM (Figure 11A). However, western analysis of SMAD2 protein expression revealed a strong induction with differentiation of primary myoblasts; increasing at day 1 and day 2 and peaking at day 3, concomitant with increased myogenin (MYOG) expression (Figure 11B). These results indicate that SMAD2 protein expression is regulated during myogenic differentiation and could have a role in this process.

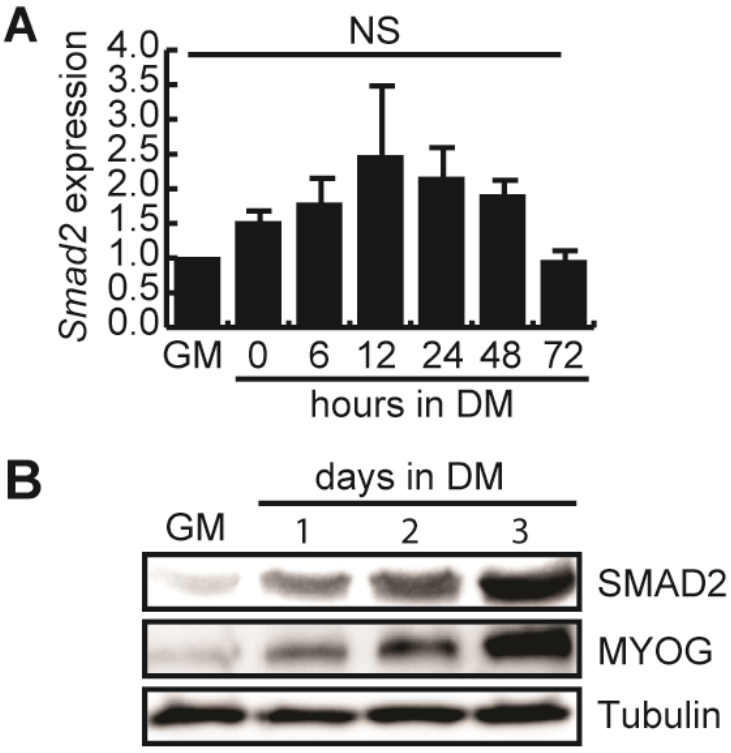

Figure 11. SMAD2 is regulated during myogenic differentiation. (A) RT-qPCR analysis of *Smad2* mRNA expression in primary myoblasts isolated from C57BL/6 mice and cultured as proliferating cells in growth medium (GM) or following induction to differentiate in low serum conditions (differentiation medium, DM) for the indicated time points (GM, DM: 0, 6, 12, 24, 48 and 72 hrs). The 0 hour time-point represents cell cultures at a high confluence which are ready for induction of differentiation. Data represents the mean and error bars represent the SEM, n=4. NS indicates there are no significant changes detected using a one-way ANOVA and Tukey's post-hoc test. **(B)** Representative western blots of SMAD2 and MYOG expression in primary myoblasts under growth conditions and after induction to differentiate in low-serum conditions (DM) for 1, 2 and 3 days. α-Tubulin is used as a loading control.

4.2 SMAD2 regulates KLF4 expression and myogenic fusion

To understand the role of SMAD2 during myogenic differentiation, primary myoblasts were isolated from the *Smad2*$^{fl/fl}$ mouse (The Jackson Laboratory) and retrovirally transduced to express a tamoxifen inducible Cre driver (CreER^{tm}). Pooled stable cells were then treated in culture with 4-OH tamoxifen (labeled TAM) for 48 hrs to induce excision of *Smad2*, or with vehicle (Veh), to generate control cells. Excision of *Smad2* was confirmed by RT-qPCR and western blot (Figure 12A, F). To assess differentiation and fusion, myoblasts were induced to differentiate for 2 days in low-serum conditions and immunocytochemistry for MyHC (green) was performed (Figure 12 B-D). Loss of *Smad2* in myoblasts did not influence the percentage of cells expressing MyHC compared to vehicle-treated myoblasts (Figure 12B). However, the fusion index (#myonuclei/#myotube), a measure of myotube size, was reduced by 45% in cells lacking *Smad2* as compared to controls (Figure 12D). To complement the fusion index, the percentage of unfused myocytes (mononucleated MyHC+ cells) was quantified and revealed that in TAM-treated cells, 30% of myocytes remain unfused, compared to 18% in controls (Figure 12E).

RT-qPCR analysis of vehicle and TAM-treated cells induced to differentiate for 2 days revealed no significant differences in expression levels of *Smad3*, *Pax7*, and *Myod1*, suggesting that differentiation occurs normally in these cells and importantly, that *Smad3* does not compensate for lack of *Smad2* expression (Figure 12F). *Myog* and *Myhc* mRNA levels were downregulated in cells lacking *Smad2*, consistent with decreased fusion (Figure 12F). Western analysis showed a decrease in MYOD, MYOG and KLF4 expression, a regulator of cell fusion, in TAM-treated myoblasts compared to control cells, also consistent with impaired terminal differentiation and fusion (Figure 12G).

Given that fusion was reduced in *Smad2*-deficient cells and KLF4 protein expression was reduced, we investigated *Klf4* as a potential target for SMAD2 transcriptional activity. Indeed, *Klf4* expression was significantly reduced in differentiated *Smad2*-deficient cells compared to controls (Figure 12H). Cells lacking *Smad2* also had reduced expression of *Npnt*, an adhesion molecule important for myoblast fusion and a known target of KLF4 (Sunadome et al. 2011) (Figure 12I). Given that loss of *Smad2* negatively affected *Klf4* levels, we next explored the *Klf4* regulatory region, and identified, using published ChIP-sequencing data coupled to motif analysis, 3 putative SMAD binding elements in the *Klf4* -500 bp promoter region (pro1: amplifies 2 Smad binding motifs at -62 and -128; pro2: amplifies one region upstream of pro1) and an enhancer region (-10 kb). Chromatin immunoprecipitation analysis using primary myoblasts differentiated for 24 hrs was performed on these 3 different *Klf4* regulatory regions and revealed SMAD2 occupancy at all 3 sites, compared to IgG control pulldown (Figure 12J). To assess whether this occupancy was functional, a reporter assay was performed by transfecting a *Klf4* promoter reporter construct, pGL3-*Klf4*(-1.6kb)-Luciferase, and a mammalian expression construct for SMAD2 in myoblasts. Myoblasts were collected 48 hours after transfection, after which luciferase activity was measured and corrected for transfection efficiency. In the presence of SMAD2, *Klf4* promoter activity was increased 5-fold as compared to the *Klf4* promoter alone (Figure 12K), and this without addition of exogenous TGFβ. Taken together, these results suggest that while SMAD2 is not required for myogenic differentiation per se, it is a transcriptional regulator of *Klf4* expression and is necessary for complete myotube maturation and myogenic fusion.

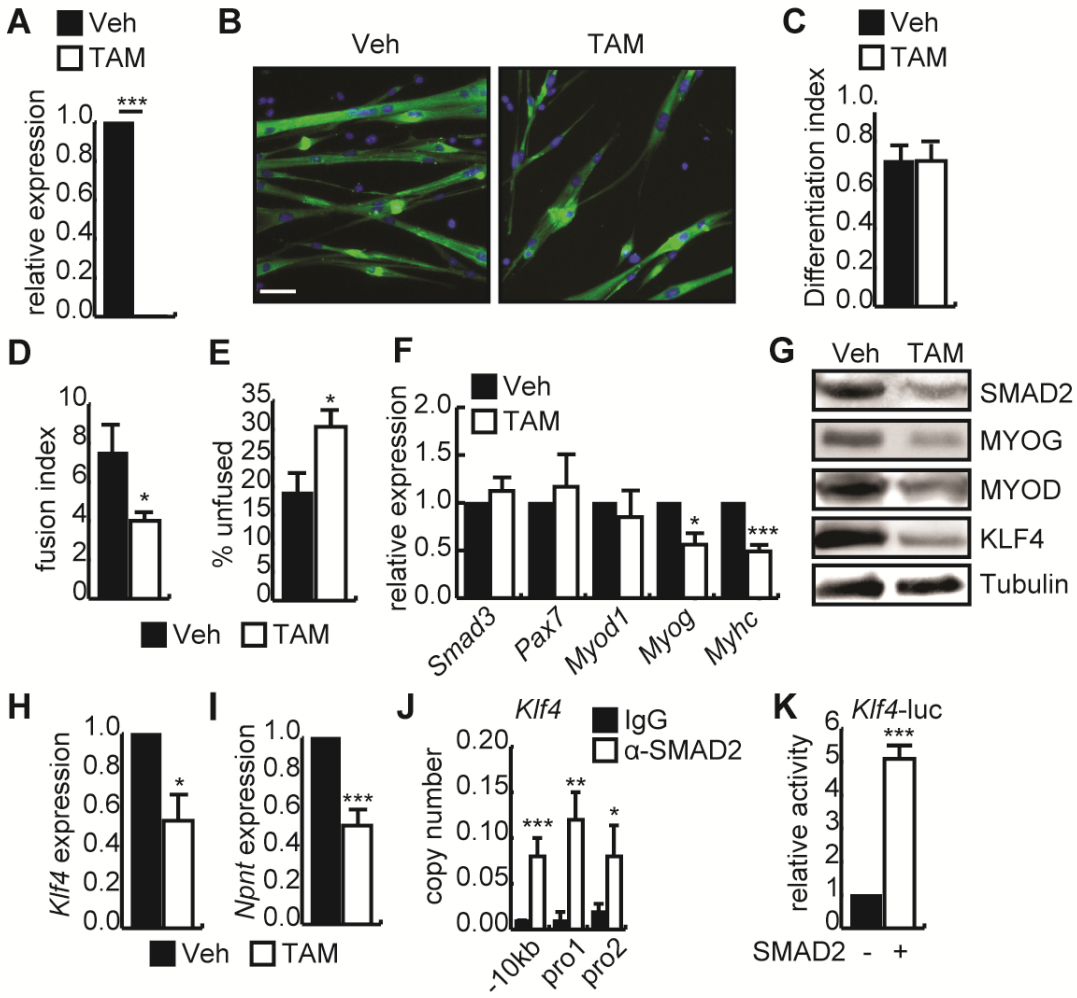

Figure 12. SMAD2 regulates KLF4 expression and myogenic fusion. Primary myoblasts isolated from *Smad2*$^{fl/fl}$ mice were retrovirally transduced to express a tamoxifen-inducible Cre recombinase (CreER) and treated with 4-OH tamoxifen (TAM) for 3 days to induce excision or with vehicle (Veh) to generate control cells. **(A)** *Smad2* mRNA expression following 4-OH tamoxifen treatment to excise *Smad2*. ***p<0.001, n=5. **(B)** Immunocytochemistry for MyHC expression in CreER primary myoblasts treated with veh or TAM and differentiated for 2 days in low serum conditions. DAPI was used to reveal nuclei; scale bar is 50 µm. **(C)** Differentiation index (#MyHC+ nuclei/ total nuclei) from cells differentiated as in (B); n=5. **(D)** Fusion index (#nuclei in MyHC+ cells with 2 or more nuclei/ # myotubes) from cells differentiated as in (B); *p<0.05, n=5. **(E)** Quantification of % unfused cells (mononucleated MyHC+ cells) from cells differentiated as in (B); *p<0.05, n=5. **(F)** RT-qPCR analysis of *Smad3*, *Pax7*, *Myod1*, *Myog and Myhc* in cells transduced and differentiated as in (B); *p<0.05, ***p<0.001, n=5. **(G)** Representative western blot of SMAD2, MYOG, MYOD and KLF4 in control and *Smad2*-deficient primary myoblasts (TAM) differentiated as in (B). **(H)** RT-qPCR analysis of *Klf4* expression in cells transduced and differentiated as in (B); *p<0.05, n=5. **(I)** RT-qPCR analysis of *Npnt* expression in cells transduced and differentiated as in (B); ***p<0.001, n=5. **(J)** Chromatin immunoprecipitation of SMAD2 occupancy of three putative SMAD motifs in the *Klf4* promoter and -10 kb upstream region performed in primary myoblasts isolated from C57BL/6 mice and differentiated for 1 day in low serum conditions. Data is shown as copy numbers (white bars) in comparison to pulldown with type-matched IgG as a control (black bars); *p<0.05, **p<0.01, ***p<0.001, n=4-6. **(K)** Reporter assay to measure *Klf4* promoter activity in the presence or absence of ectopic SMAD2, shown relative to reporter alone and corrected for transfection efficiency. ***p<0.001, n=4.

4.3 *In vivo* loss of *Smad2* impairs regeneration

To further understand the consequence of loss of SMAD2 *in vivo*, and since *Smad2*-null animals are embryonic lethal, we generated a conditional *Smad2* knockout murine model where *Smad2* is excised in PAX7-expressing satellite cells. *Smad2*$^{fl/fl}$*Pax7*$^{CreER/wt}$ males were bred to *Smad2*$^{fl/fl}$*Pax7*$^{wt/wt}$ females to yield *Smad2*$^{fl/fl}$*Pax7*$^{CreER/wt}$ experimental animals (*Smad2*^{cKO}) and *Smad2*$^{fl/fl}$*Pax7*$^{wt/wt}$ control littermates (WT) (Figure 13A). Excision of *Smad2* in satellite cells was achieved with i.p. tamoxifen injections for 5 days. A unilateral cardiotoxin (CTX) injury was performed 7 days post-TAM treatment and both tibialis anterior (TA) muscles were dissected, weighed and flash frozen 7 days post-injury (Figure 13B). RT-qPCR analysis showed *Smad2* knockdown in the *Smad2*^{cKO} myoblasts compared to WT controls (Figure 13C), and this was also shown as a decrease in the percentage of cells expressing both SMAD2 and PAX7 by performing immunohistochemistry on muscle sections (Figure 13D). Histological analysis of muscle sections stained with hematoxylin and eosin (H&E) revealed no significant change in average fiber XSA between genotype in the uninjured muscle (Figure 13E, F). However after CTX injury, muscle from the *Smad2*^{cKO} had impaired regeneration, demonstrated by a reduction in the average muscle fiber XSA, as compared to WT injured control muscle (Figure 13E, F). While cells expressing PAX7 were increased after CTX injury in WT animals, as expected, we did not observe any significant differences in the percentage of PAX7-expressing cells, by immunohistochemistry, when comparing uninjured or injured WT and *Smad2*^{cKO} muscle (Figure 13G). To further quantify the differences between injured WT and *Smad2*^{cKO} muscle, the distribution of regenerated fibers was analyzed. Indeed, a trend towards increased small fibers (<500 μm^2) and fewer large fibers (>800 μm^2) was revealed for the *Smad2*^{cKO} muscles compared

to WT, suggesting a higher frequency of very small fibers (Figure 13H). Taken together, these results suggest that SMAD2 is required for efficient muscle regeneration.

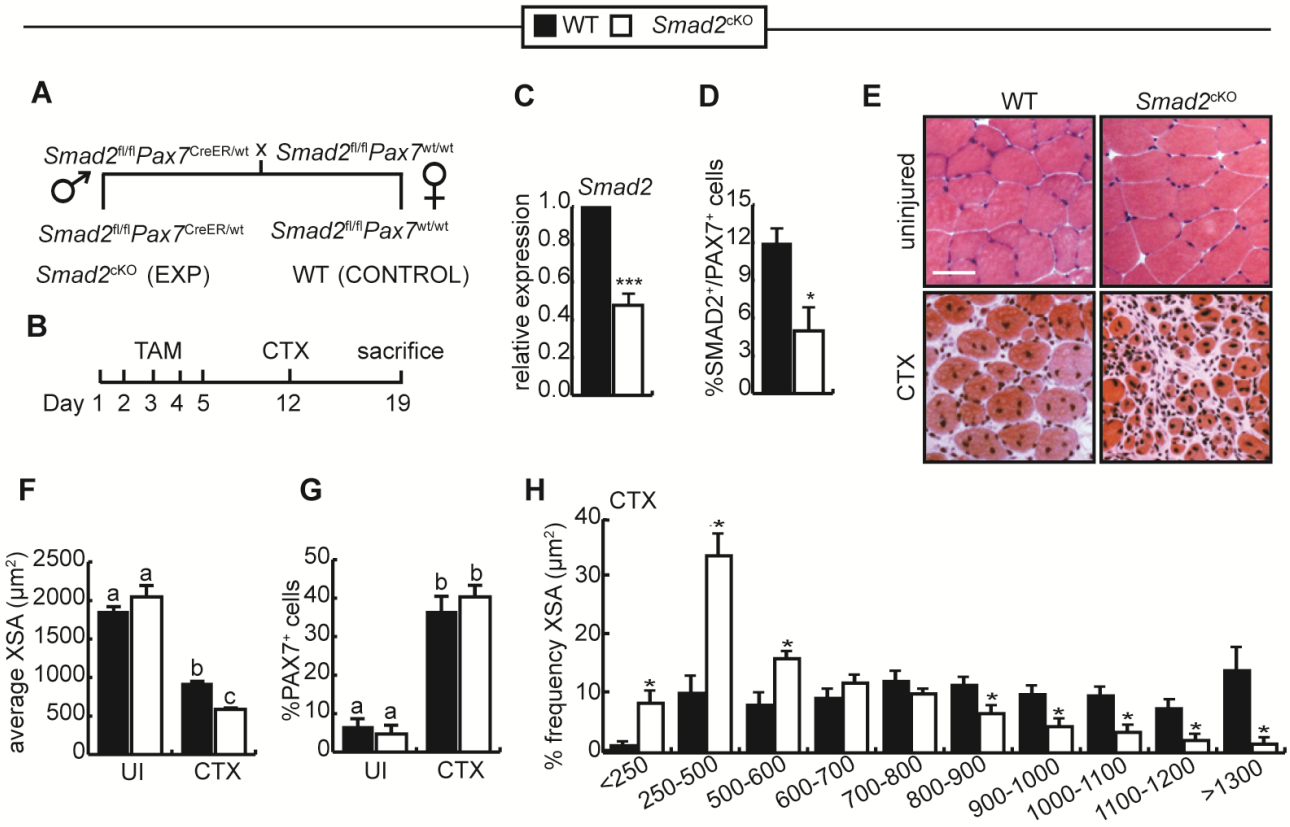

Figure 13. SMAD2 is required for efficient muscle regeneration. (A) Schematic representation of the breeding strategy used to obtain WT control and *Smad2*^{cKO} animals. **(B)** Schematic representation of the regeneration experiment including *in vivo* excision of *Smad2* by 5-daily i.p. tamoxifen injections, followed by cardiotoxin (CTX) injury and sacrifice 7 days post-injury. **(C)** *Smad2* mRNA expression in satellite cells isolated from WT and *Smad2*^{cKO} mice 2 weeks after treatment with tamoxifen for 5 days. ***p<0.001, n=4. **(D)** Percentage of SMAD2+/PAX7+ cells relative to total nuclei in muscle sections taken from mice treated with tamoxifen as in (B). **(E)** Representative images of muscle sections from WT and *Smad2*^{cKO} mice whose right tibialis anterior (TA) was not injured (UI) and the left TA was injured with cardiotoxin (CTX) and allowed to repair for 7 days. Scale bar = 50 µm. **(F)** Average cross-sectional area (XSA) of muscle fibers in WT and *Smad2*^{cKO} mice injured as in (B). **(G)** Percentage of PAX7+ cells relative to total nuclei from WT and *Smad2*^{cKO} animals, injured (CTX) or uninjured (UI) as in (E). For F-G, means indicated with different letters are significantly different from one another at a minimum cut-off of p<0.05, n= 4 pairs. **(H)** Percent frequency of XSA of muscle from (E). For all graphs data represents the mean and error bars represent the SEM; *p<0.05, ***p<0.001, n=4 pairs (data combined for males and females is shown).

4.4 Loss of SMAD2 *in utero* leads to smaller fibers

In the regeneration experiment, where *Smad2* was excised in adult mice with tamoxifen injections only 2 weeks prior to tibialis anterior harvest, we did not observe a notable change in myofiber size in uninjured WT and *Smad2*^{cKO} muscle. To investigate the effect of loss of *Smad2* during the post-natal period, a phase of extensive satellite cell proliferation, *Smad2*$^{fl/fl}$*Pax7*$^{wt/wt}$ females were bred to *Smad2*$^{fl/fl}$*Pax7*$^{CreER/wt}$ males and *Smad2* was excised *in utero* by gavage of the pregnant dams at embryonic day 15.5 (E15.5) with a single dose of tamoxifen (1.5mg/20g body weight). Pups were subsequently sacrificed at post-natal day 21 (P21) and tibialis anterior muscles were dissected and flash frozen for histological analysis and PAX7 immunohistochemistry. The remaining hindlimb muscles were digested for satellite cell isolation to confirm excision of SMAD2. Western analysis of satellite cells isolated from *Smad2*^{cKO} and WT animals confirmed the knockdown of SMAD2 expression (Figure 14A). Hematoxylin and eosin (H&E) staining and analysis of the XSA revealed smaller myofibers at P21 in *Smad2*^{cKO} muscle compared to WT (Figure 14B, C). Further analysis demonstrated that the total fiber numbers trended towards an increase in *Smad2*^{cKO} muscles compared to WT, however this did not reach statistical significance (Figure 14D). Immunohistochemistry revealed no difference in the percentage of PAX7 positive cells between *Smad2*^{cKO} and WT muscle sections at P21, suggesting that the decrease in fiber caliber is not due to decreased satellite cell numbers (Figure 14E). These results are consistent with the impaired regeneration phenotype observed in Figure 13 and with the impaired terminal differentiation and fusion observed in culture.

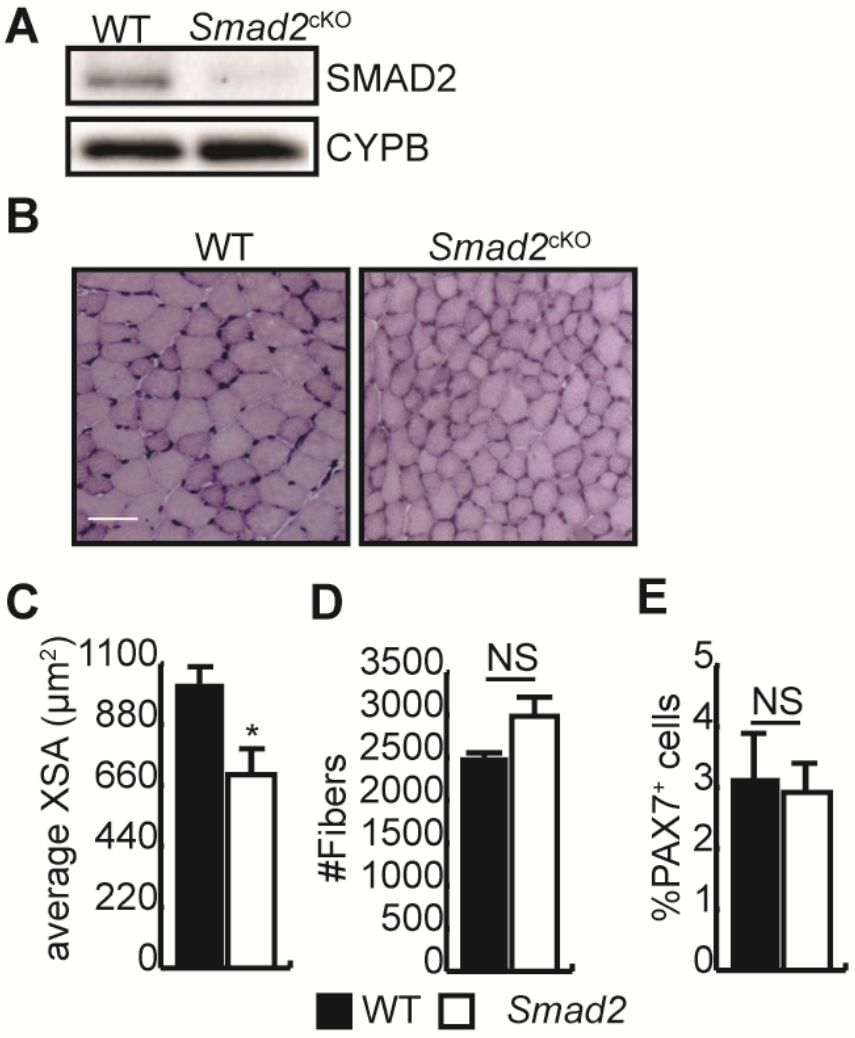

Figure 14. Loss of SMAD2 in utero leads to smaller fibers. (A) *Smad2*$^{fl/fl}$*Pax7*$^{wt/wt}$ pregnant dams bred to *Smad2*$^{fl/fl}$*Pax7*$^{CreER/wt}$ males were gavaged with tamoxifen (1.5mg/20g body weight) at embryonic day 15.5 (E15.5). Pups were sacrificed at post-natal day 21 (P21); tibialis anterior and hind limb muscles were collected and animals genotyped. Western analysis of SMAD2 and CYPB (loading control) from satellite cells isolated from P21 WT and *Smad2*^{cKO} mice in growth conditions. **(B)** Hematoxylin and eosin (H&E) staining of muscle sections from WT and *Smad2*^{cKO} mice at P21; n=3 pairs (data combined for males and females is shown), scale=50um. **(C)** Average cross-sectional area (XSA) calculated from muscle sections as in (A); *p<0.05, n=3 pairs. **(D)** Total number of fibers in WT and *Smad2*^{cKO} pups from muscle sections as in (A); p=0.056. **(E)** Percentage of PAX7 positive cells from immunohistochemistry of muscle sections as in (A). Graphs represent the mean and the error bars are the SEM. NS= non significant.

4.5 Forced expression of SMAD2 enhances myoblast fusion

Given that loss of SMAD2 *in vivo* led to i) smaller myofiber cross sectional area at P21; (ii) impaired regeneration after acute injury, and (iii) decreased fusion in myotubes, we sought to evaluate the role of SMAD2 in myogenic fusion by creating stable myoblast cell lines overexpressing SMAD2. C2C12 myoblasts were retrovirally transduced to express SMAD2 or an empty vector control (pLPCX) and induced to differentiate for 4 days in low-serum conditions (Figure 15D). Overexpression of SMAD2 was confirmed by western and RT-qPCR analysis (Figure 15A, C). RT-qPCR analysis revealed an increase in *Myog* and *Klf4* expression in SMAD2 overexpressing cells compared to control cells after 1 day in differentiation conditions, consistent with enhanced fusion (Figure 15B). Further, at this time-point, *Smad3* and *Myod1* expression did not change between SMAD2 overexpressing and control cells. Western analysis confirmed the RT-qPCR results with SMAD2, MYOG and KLF4 all upregulated in SMAD2 overexpressing cells compared to controls (Figure 15C). Immunocytochemistry for MyHC revealed increased differentiation and larger myotubes in cells overexpressing SMAD2, as calculated by the differentiation (#MyHC+ nuclei/#total nuclei) and fusion indices (#nuclei/#myotubes) (Figure 15E, F). The enhanced fusion observed in SMAD2 overexpressing cells was not due to a significant increase in cell numbers as nuclei per area was not changed compared to pLPCX controls (Figure 15G). Consistent with robust fusion and KLF4 upregulation, *Npnt* levels were increased in SMAD2-overexpressing cells compared to controls after 4 days in differentiation conditions (Figure 15H).

Next, we sought to determine whether the increased differentiation and fusion observed with forced expression of SMAD2 would require activation by TGFβ signalling, whose ligand is likely present in the culture medium. To this end, we retrovirally transduced C2C12 cells to

express full length SMAD2 (SMAD2), a SMAD2 C-terminal mutant (SMAD2ΔSSMS; missing the two C-terminal serines required for canonical TGFβ signalling) or a control empty vector (pLPCX) and evaluated differentiation after 4 days. We find that the SMAD2ΔSSMS mutant is able to enhance myogenic differentiation and fusion similar to full length SMAD2 (Figure 15I, J) without impacting cell numbers (Figure 15K). These results suggest that the stimulation of terminal myogenic differentiation and fusion by SMAD2 does not depend on the presence of the C-terminal SSMS motif and therefore classical TGFβ signalling pathway.

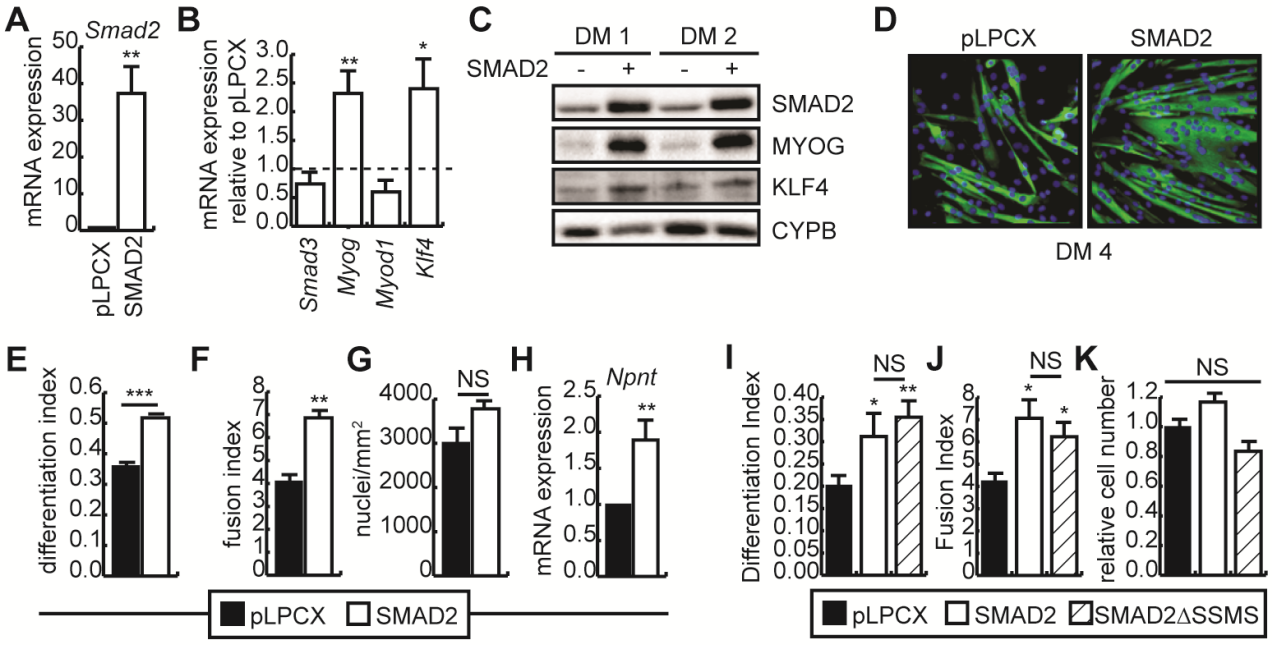

Figure 15. Overexpression of SMAD2 enhances myogenic fusion. **(A)** RT-qPCR analysis of *Smad2* expression in C2C12 myoblasts retrovirally transduced to express SMAD2 or with empty virus (pLPCX) and differentiated for 24 hrs. **p<0.01, n=6. **(B)** RT-qPCR analysis of *Smad3*, *Myog*, *Myod1* and *Klf4* in myoblasts transduced as in (A) and differentiated for 24 hrs. Data for SMAD2-overexpressing cultures is shown as the means relative to controls, indicated by the hatched line. *p<0.05, **p<0.01, n=4-8. **(C)** Western analysis of SMAD2, MYOG and KLF4 expression in myoblasts transduced as in (A) after induction to differentiate for 1 day (DM 1) or two days (DM 2). Cyclophilin B (CYPB) is used as a loading control. **(D)** Indirect immunocytochemistry for MyHC expression in cells transduced as in (A) and induced to differentiate for 4 days. DAPI counterstains the nuclei. **(E)** Differentiation index (#MyHC+ nuclei/ total nuclei) from cells differentiated as in (D). ***p<0.001, n=3. **(F)** Fusion index (# nuclei found in MyHC+ cells with 2 or more nuclei/ # myotubes) from cells differentiated as in (D). **p<0.01, n=3. **(G)** Cell culture density as cells per mm^2 from cultures transduced and differentiated as in (D). NS = not significant. **(H)** RT-qPCR analysis of *Npnt* expression in cells transduced as in (A) and differentiated for 4 days. **p<0.01, n=6. **(I)** Differentiation index (# nuclei in MyHC+ cells/ total nuclei) from C2C12 cells transduced to express empty vector control (pLPCX), SMAD2 or a truncated SMAD2 lacking the C-terminal SSMS motif (SMAD2ΔSSMS) and differentiated for 4 days in low serum conditions. *p<0.05, **p<0.01, n=4. **(J)** Fusion index (# nuclei found in MyHC+ cells with 2 or more nuclei/ # myotubes) from cells differentiated as in (I). *p<0.05, n=4. **(K)** Cell culture density as cells per mm^2 from cultures transduced and differentiated as in (I). NS = not significant. Graphs represent the mean and error bars represent the SEM.

4.6 *Smad2* is required for KLF4-induced *Npnt* expression during myogenic fusion

Given that *Klf4* is a transcriptional target of SMAD2 in myoblasts and that fusion is enhanced in cells overexpressing SMAD2, we sought to force KLF4 expression in myoblasts lacking SMAD2 and determine its effect on fusion. *Smad2*$^{fl/fl}$ primary myoblasts expressing CreER and treated with vehicle (WT) or 4-OH tamoxifen (*Smad2*^{cKO}) were retrovirally transduced to express KLF4 or an empty vector control (EV). These primary myoblasts were induced to differentiate in low-serum conditions for 2 days after which differentiation and fusion was assessed by immunocytochemistry for MyHC. RT-qPCR analysis confirmed the knockdown of *Smad2* in *Smad2*^{cKO} expressing EV and KLF4 cells (Figure 16D). Overexpression of *Klf4* was also confirmed by RT-qPCR in WT and *Smad2*^{cKO} myoblast transduced with KLF4, with levels being increased 5.5-fold in WT KLF4 (white bar) and 4 fold in *Smad2*^{cKO} KLF4 (grey bar) compared to control (black bar) (Figure 16D). Quantification of the differentiation index revealed no differences between WT and *Smad2*^{cKO} cells expressing empty vector or KLF4 (Figure 16B). However, forced expression of KLF4 in WT cells resulted in a 5-fold increase in the fusion index (#nuclei/#myotubes), compared to WT EV cells. As shown previously, *Smad2*^{cKO} EV myoblasts had a 53% decrease in myotube size (quantified as the fusion index) compared to WT EV cells (Figure 16A, C). Similarly, a 46% decrease in the fusion index was observed in *Smad2*^{cKO} cells overexpressing KLF4 (Figure 16C). Consistent with the reduced fusion observed in *Smad2*^{cKO} EV myoblasts, RT-qPCR analysis revealed a significant reduction in *Klf4* expression at day 2 of differentiation, compared to WT EV cells (Figure 16D). Although elevated *Klf4* levels enhanced fusion in WT KLF4 cells, fusion remained impaired when KLF4 expression was forced in *Smad2*^{cKO} cells, even with a 4-fold increase in *Klf4* expression, as compared to WT EV control cells (Figure 16D). We then evaluated *Npnt* levels, a KLF4 target

gene, 6 hours after induction to differentiate in low-serum conditions a time-point suitable for detection of genes in early fusion. Forced expression of KLF4 in WT cells resulted in a modest but significant increase in *Npnt* expression compared to WT EV controls (Figure 16D). *Npnt* expression was significantly decreased by 75% in *Smad2*^{cKO} EV cells and by 65% in *Smad2*^{cKO} cells overexpressing KLF4 compared to WT EV controls (Figure 16D). These results suggest that *Smad2* is required for the expression of *Npnt* downstream of KLF4 and efficient fusion. Next, we looked at the *Npnt* gene regulatory region and performed a motif analysis for putative KLF4 and SMAD binding sites. Interestingly, we found a region located at -12 kb from the *Npnt* gene with MYOD binding and H3K27Ac histone marks, corresponding to an active enhancer. Following single site motif analysis we found 3 KLF4 binding sites and one SMAD binding site (Figure 16E). Within the +650 bp region downstream of the *Npnt* transcription start site, we found 3 KLF4 binding sites at close proximity to one another (157 bp) but no putative SMAD binding site (Figure 16E). Primers were designed to amplify 3 specific regulatory regions of the *Npnt* gene: pro1 which contains 1 KLF4 motif, pro2 which has 2 KLF4 motifs and the -12 kb region which contains 3 KLF4 motifs and 1 SMAD motif (Figure 16D). Chromatin immunoprecipitation experiments revealed that SMAD2 and KLF4 occupy all 3 *Npnt* regulatory regions examined, under differentiation conditions in C2C12 myoblasts. Taken together these results suggest that *Smad2* is required for the regulation of *Npnt* by KLF4 during myogenic fusion, as the increase in myotube size generated by forced expression of *Klf4* is lost in cells lacking *Smad2*.

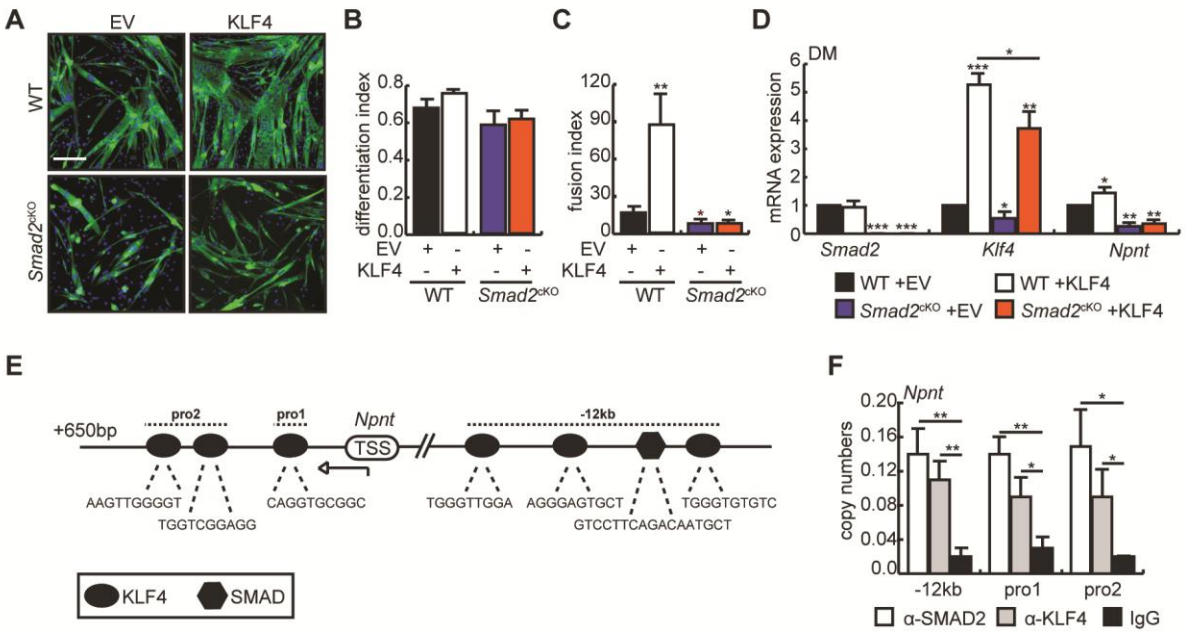

Figure 16. Forced expression of KLF4 does not promote fusion in SMAD2-deficient myoblasts. (A) *Smad2*$^{fl/flCreER}$ primary myoblasts treated with 4-OH tamoxifen (*Smad2*^{cKO}) to excise *Smad2* or vehicle (WT) were retrovirally transduced to express KLF4 or pMK-Ctl (empty vector, EV) and induced to differentiate for 2 days in low-serum conditions. Immunocytochemistry for myosin heavy chain was performed and representative images are shown. DAPI is used to reveal the nuclei. n=4, scale bar=100um. **(B)** Quantification of the differentiation index (#Myosin heavy chain+ nuclei/#total nuclei) of cells cultured and transduced as in (A). n=4. **(C)** Fusion index (#nuclei/myotube) of cells cultured, transduced and differentiated as in (A). n=4. **(D)** RT-qPCR analysis of *Smad2*, *Klf4* and *Npnt* in cells transduced as in (A) and induced to differentiate for 6 hrs in low-serum conditions. *p<0.05, ** p<0.01 *** p<0.001, n=4. **(E)** Schematic representation of 3 *Npnt* regulatory regions (-12 kb, pro1 and pro2) containing KLF4 and SMAD binding sites. **(F)** Chromatin immunoprecipitation showing SMAD2 and KLF4 occupancy of 2 regions in the *Npnt* promoter and -12 kb upstream region performed in C2C12 cells differentiated for 1 day in low serum conditions. Data is shown as copy numbers (diagonal and horizontal bars) in comparison to pulldown with type-matched IgG as a control (black bars). *p<0.05, **p<0.01, ***p<0.001, n=3. Graphs represent the mean and the error bars represent the SEM.

CHAPTER 5: DISCUSSION

5.1 Summary of findings

Although sharing high sequence similarity, SMAD2 and SMAD3 have been shown to be functionally different in many contexts. Previously, our laboratory discovered that SMAD3 can disrupt C/EBPβ binding of target promoters in models of adipogenesis and osteoblastogenesis, leading to a block or enhancement of differentiation in these cell types, respectively. Results herein demonstrate that SMAD3 expression is upregulated by RA treatment in myoblasts in both growth and differentiation conditions and leads to enhanced differentiation through this same mechanism. Addition of RA to TGFβ-treated cells partially rescued differentiation and fully restored fusion. We show that RA treatment disrupts C/EBPβ occupancy of the *Pax7* and *Smad2* promoters, and we therefore describe a positive role for RA-induced SMAD3 in myogenic differentiation.

In addition to a pro-myogenic role for SMAD3, we demonstrate that C/EBPβ is a target of the TGFβ pathway, and can stimulate the expression of SMAD2, adding more insights into the role of C/EBPβ as a negative regulator of myogenesis. Indeed, TGFβ treatment upregulated C/EBPβ expression, while RA treatment decreased C/EBPβ back to control levels, consistent with autoregulation of the *Cebpb* promoter by C/EBPβ (Niehof et al. 2001). Moreover, loss of C/EBPβ in primary myoblasts partially protected these cells from the inhibition of myogenesis caused by TGFβ, suggesting that C/EBPβ is a potent mediator of the anti-myogenic effects of TGFβ.

To further study the function of SMAD2 *in vivo* during myogenesis, we created a novel murine model where *Smad2* is excised in muscle satellite cells. We demonstrate that loss of *Smad2* leads to impaired myoblast fusion without affecting differentiation in culture and a regeneration defect *in vivo* after an acute injury. Consistent with these findings, loss of *Smad2 in*

utero also decreased the muscle fiber calibre in the post-natal animal. In accordance, forced expression of SMAD2 enhanced myoblast fusion and increased KLF4 and myogenin expression. Our in culture SMAD2 gain and loss of function experiments revealed that *Klf4* is a SMAD2 target, with SMAD2 occupying and activating the *Klf4* promoter. Forced expression of KLF4 enhanced fusion in WT myoblasts, but fusion was completely impaired in both *Smad2*^{cKO} EV and *Smad2*^{cKO} cells overexpressing KLF4, indicating that SMAD2 is required for myoblast fusion, even in the presence of a fusogenic factor. In addition, we show that SMAD2 is a regulator of *Npnt* and that in the absence of SMAD2, KLF4 is unable to upregulate *Npnt* which is necessary for myogenic fusion. We therefore identify a positive role for RA-activated SMAD3, and unexpectedly, a positive role for SMAD2 in terminal myogenic differentiation/fusion and regeneration.

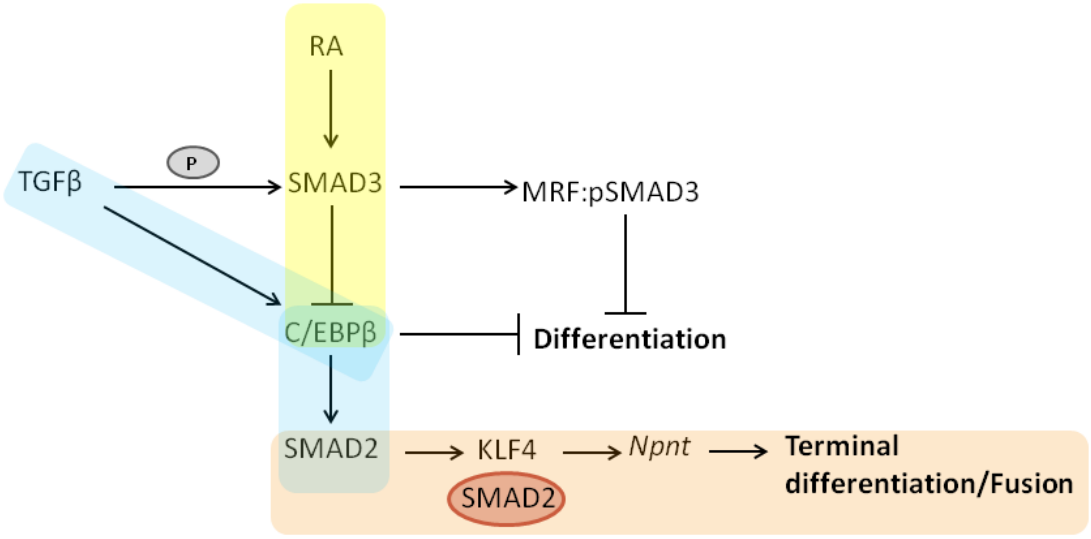

Figure 17. Contributions model figure. It is well known that TGFβ inhibits myogenesis through SMAD3 which disrupts MYOD and MEF2 transcriptional complexes. This canonical TGFβ-mediated phosphorylation of SMAD3 arm is dominant and active in presence of TGFβ signals and inhibits myogenic differentiation. However, in the presence of retinoic acid (RA), SMAD3 upregulation, which is independent of its C-terminal phosphorylation, has pro-myogenic effects by decreasing C/EBPβ expression and disrupting its activity (yellow box). TGFβ upregulates C/EBPβ and forced expression of C/EBPβ increased SMAD2 (blue box). SMAD2 expression is upregulated during myogenic differentiation and forced SMAD2 expression results in increased terminal differentiation and fusion. SMAD2 regulates *Klf4* and is required for the *Klf4*-induced expression of its target *Npnt* and productive fusion (orange box). This work uncovers a pro-myogenic role for RA-induced SMAD3 and describes a novel regulatory mechanism where SMAD2 regulates myogenic fusion through *Klf4* and *Npnt*.

5.2 Retinoic acid-induced SMAD3 has pro-myogenic actions in myoblasts

Our laboratory has previously shown that in osteoblastogenesis and adipogenesis, RA treatment induced both the expression and nuclear localization of SMAD3, without affecting SMAD2 expression (Dingwall et al. 2011; Marchildon et al. 2010). In studying the regulation of SMAD3 expression by RA, *in silico* analysis of the mouse *Smad3* promoter region revealed no retinoic acid response element (RARE) for binding of RAR-RXR heterodimers to activate *Smad3* transcription. However, we found that RAR did occupy a RARE site in the intronic region between exon 3 and exon 4 of the *Smad3* gene, and this could offer a way by RA to enhance *Smad3* transcription. Further examination of potential binding sites for the transcription factors RAR-RXR on the *Smad3* regulatory region revealed 10 binding sites within the -10 kb region; including sites within the promoter region (+/-1 kb) (Kwon et al. 2012). ChIP analysis followed by a reporter assay would confirm if these RAR-RXR binding sites are functional in activating *Smad3* transcription.

Interestingly, our prior findings revealed that the increase in SMAD3 expression by RA treatment did not correlate with changes in SMAD3 C-terminal phosphorylation levels or increased SMAD2 protein expression. This is consistent with results shown here, where we observed increased SMAD3, but not SMAD2, protein expression in myoblasts subjected to a 48 hour RA treatment. Additionally, RA treatment had no impact on SMAD3 C-terminal phosphorylation (serine 423/425) in our experiments. However, RA increased phosphorylation of serine 213 in the linker region and expression of SMAD4 in myoblasts (unpublished observations). Indeed, it is suggested that the linker phosphorylation status of SMAD3 has different effects on its activity (Mori et al. 2004; Matsuzaki et al. 2009; Matsuzaki 2011; Kamato et al. 2013; Sapkota et al. 2006). One such example has been provided in cancer; where C-

terminally phosphorylated SMAD2 and SMAD3 inhibited proliferation of epithelial cells while linker phosphorylation of SMAD2 and SMAD3 by CDK4 promoted cell growth (Matsuzaki 2011). It is possible that RA could drive phosphorylation or another post-translational modification at a site(s) other than the classical C-terminal serines targeted by the TGFβ receptor, which could modify SMAD3 actions into a pro-myogenic role. Perhaps one of these SMAD3 post-translational modifications could act as a trigger switch to change the function of SMAD3 from an anti-myogenic role in the presence of TGFβ to a pro-myogenic one. Our results suggest that the RA-induced activation of SMAD3 is different from the TGFβ-induced activation of SMAD3, and this could influence function of SMAD3 in myoblasts. It would therefore be interesting to conduct a mass-spectrometry experiment in myoblasts to determine potential post-translational modifications of SMAD3 in the presence and absence of RA that differ from treatment with TGFβ.

We believe that SMAD3 activated by RA has pro-myogenic actions, by displacing the transcription factor C/EBPβ from the *Pax7* promoter, and inducing differentiation of myoblasts (Marchildon et al. 2012). However, in the presence of TGFβ, SMAD3 could still relay the anti-myogenic effects of this signalling pathway. Indeed, although it is apparent that the TGFβ-phosphorylated SMAD3 branch of the signalling pathway is dominant and potently anti-myogenic, our study and other groups have shown that SMAD3 has pro-myogenic potential during myogenic differentiation in the presence of RA and also in the absence of TGFβ activation (Lamarche, 2015; Ge, 2011; Ge et al. 2012; Mullen et al. 2011). As many studies focus on the C-terminal phosphorylation state of SMAD2 and SMAD3 almost as sole parameter when studying myogenic differentiation, it is important to note that SMAD proteins may retain

function even without being activated by TGFβ, and for the case of SMAD2, without having DNA-binding activities itself.

It is unknown whether C-terminally phosphorylated SMAD3 can still interfere with the transcriptional activities of C/EBPβ. Experiments using full length GST-tagged SMAD3 or the SMAD3ΔSSVS mutant and pulldown with C/EBPβ in the presence or absence of TGFβ could add knowledge into the conditions governing the physical interaction between C/EBPβ and SMAD3. This notion is in line with the literature showing that TGFβ-phosphorylated SMAD3 inhibits myogenesis by forming inhibitory complexes with MEF2 and MYOD (Liu, Kang, and Derynck 2004; Liu, Black, and Derynck 2001). However, as demonstrated by Mullen et al., MYOD, a master positive regulator of myogenesis, recruits SMAD3 to its targets in myotubes in the presence of TGFβ, which goes against the known anti-myogenic role of TGFβ-activated SMAD3 during myogenic differentiation (Mullen et al. 2011). If SMAD3 can inhibit MYOD, then why would it be recruited by MYOD and found co-occupying sites bound by MYOD in myotubes? Perhaps in late differentiation, MYOD recruits SMAD3 to its targets to limit differentiation. Prolonged TGFβ treatment, such as in the Liu study, could inhibit MYOD activity while shorter TGFβ exposure could ensure recruitment of SMAD3 to MYOD target genes and initiation of differentiation. Given that TGFβ was added at the end of myotube differentiation in the Mullen study, when MYOD has already activated MYOG for example, and cells are largely refractory to the inhibition of differentiation by TGFβ, MYOD could recruit SMAD3 to targets needed for terminal differentiation. It remains to be discovered whether MYOD can also recruit SMAD2 to specific targets given that the SMADs share a common binding motif, and specifically if those genes targets are involved in fusion.

5.3 Retinoic acid treatment partially rescues TGFβ-induced inhibition of myogenesis

Prior to our results, RA had previously been identified as being able to counteract the anti-myogenic effect of TGFβ; however, the mechanism of action of RA remained unknown (Krueger and Hoffmann 2010). TGFβ can increase the activation of a SMAD3-dependent luciferase reporter, but addition of RA, despite upregulating SMAD3 expression, had no effect on this activation, suggesting that the positive effect of RA is not through inhibition of classical TGFβ-activated SMAD3 (Krueger, 2010). Our results also show that RA can antagonize TGFβ-mediated inhibition of myogenesis without affecting the phosphorylation of SMAD3 C-terminal serines but by increasing overall SMAD3 levels. This finding is consistent with our laboratory's previous finding showing that upon RA treatment, SMAD3 expression and nuclear accumulation, was increased without effects on C-terminal serine phosphorylation or SMAD2 expression.

One interesting finding of our work was the reduction of TGFβ-induced SMAD2 phosphorylation upon addition of RA, with no effect on SMAD3 C-terminal phosphorylation. This result suggests a differential mechanism for SMAD regulation by RA and led us to believe that decreasing pSMAD2 levels would be beneficial for myogenesis as these cells were relieved from the TGFβ-induced blockade of differentiation. It is possible that RA induces the expression of a protein phosphatase that specifically targets C-terminal serines of SMAD2 or that it enhances SMAD2 linker phosphorylation, proposed to decrease SMAD transcriptional activity. PPM1A and SCP1 have been identified as specific SMAD2/3 C-terminal and linker phosphatases, respectively (Wrighton et al. 2006). Although mechanisms of differential regulation of SMAD phosphorylation have not yet been fully uncovered, PP2A was found to be a SMAD3-specific phosphatase under hypoxic conditions (Heikkinen et al. 2010). To our

knowledge, PP2A is the only SMAD3-specific phosphatase, although it remains possible that a SMAD2-specific phosphatase exists.

5.4 TGFβ treatment upregulates C/EBPβ

Although TGFβ upregulated C/EBPβ expression in C2C12 myoblasts, we did not observe the same robust increase of C/EBPβ expression in primary myoblasts. As the course of differentiation differs in primary myoblasts (2 days) compared to C2C12 cells (4 days), the longer exposure to TGFβ in C2C12 cells could enhance C/EBPβ expression after the 4 day timepoint. Additionally, as cell confluency greatly affects differentiation and ability of TGFβ to inhibit myogenesis, adding TGFβ to over confluent cells that have already started to express myogenin would lessen the effects of TGFβ. The concentration used in these experiments also differed; a concentration of 1ng/ml was used for C2C12 cells compared to 5ng/ml in primary myoblasts. In both C2C12 and primary myoblasts, TGFβ treatment did not affect *Smad2* expression at the end-point of differentiation. *Pax7* expression, while being upregulated robustly in C2C12 cells, was only modestly stimulated in primary myoblasts upon TGFβ treatment, suggesting C2C12 cells are more sensitive to TGFβ treatment. Although C/EBPβ was not increased by TGFβ treatment of primary myoblasts, cells lacking C/EBPβ were partially protected from the anti-myogenic effects of TGFβ. Indeed, primary myoblasts express higher levels of C/EBPβ than C2C12 myoblasts which may account for the difference observed. The upregulation of C/EBPβ by TGFβ treatment may serve to amplify TGFβ signalling in certain contexts as we believe that C/EBPβ is only part of the anti-myogenic arsenal of TGFβ. Given the partial rescue observed in our TGFβ-treated *Cebpb*^{cKO} myoblasts, it would suggest that C/EBPβ is not the main mediator of TGFβ signalling and that TGFβ exerts its actions through other mechanisms, which may be independent of SMAD2 and SMAD3. However, one must consider

the incomplete excision of *Cebpb* in these experiments, which could contribute directly to a less than complete rescue of differentiation. Experiments using retroviral CreER transduction of *Cebpb*$^{fl/fl}$ primary myoblasts to increase *Cebpb* excision or CRISPR-Cas9 technology in WT primary myoblasts would clarify this finding.

Interestingly, we show that TGFβ treatment completely abolishes SMAD3 expression in primary myoblasts differentiated for 2 days, as was observed in human osteoarthritic chondrocytes (Baugé, 2010). In chondrocytes, TGFβ treatment increased SMAD3 protein and RNA levels after 1 hour; however, prolonged exposure to TGFβ lead to decreased SMAD3, decreased TGFβRI-II and increased the expression of the inhibitory SMAD, SMAD7. This could explain why SMAD3 levels are decreased in our experiment, where TGFβ is present in the culture for 2 days.

C/EBPβ has previously been shown to mediate TGFβ actions through SMADs in other systems. For example, during breast cancer progression, TGFβ induces growth inhibition through the formation of SMAD3 complexes with FoxO and C/EBPβ, which induce cell-cycle inhibitors and repress cell cycle progression genes such as c-Myc (Johansson et al. 2013). However, loss of C/EBPβ, by oncomiR miR-155-mediated targeting, which is overexpressed in breast cancer, switches TGFβ actions from growth inhibition to invasion by inducing epithelial-to-mesenchymal transition (EMT), a hallmark of metastasis (Johansson et al. 2013).

5.5 New insights on SMAD2 and SMAD3 as effectors of TGFβ signalling

It was long considered that SMAD2 and SMAD3 were actively shuttling between the cytoplasm and nucleus at basal state, and would accumulate in the nucleus only upon activation by TGFβ. However, Liu et al. recently found that SMAD3 was mostly located in the nucleus

while SMAD2 was found in the cytoplasm at basal state in human and mouse embryonic stem cells (Liu, 2016). Surprisingly, SMAD3 nuclear localization was found to be independent of SMAD4 levels and C-terminal phosphorylation by TGFβ. It is thought that the location of SMAD3 in the nucleus makes it less sensitive to TGFβ signals from the extracellular space and that SMAD2 would be the main effecter of TGFβ in these cells. In this study, SMAD3 nuclear function was found to be required for neuronal specification and differentiation, which was strictly a function of SMAD3 and not SMAD2. This study concluded that because of SMAD2's availability in the cytoplasm, in these cells, it is the main TGFβ effector protein as it is more sensitive to TGFβ signals.

The muscle phenotype was investigated in a *Smad3* knockout mouse, targeting exon 8 and producing a truncated protein lacking the last 89 amino acids of the C-terminal tail that includes the SSVS motif for TGFβ activation (Ge et al. 2011; Yang 1999). This truncated mutant SMAD3 protein was not responsive to TGFβ. Since it is missing part of MH2 domain, this mutant SMAD3 protein would not be expected to inhibit myogenesis through inhibition of MEF2 and MYOD nor be phosphorylated by the TGFβ receptor complex (Liu, Kang, and Derynck 2004; Liu, Black, and Derynck 2001). Since SMAD3 is an important mediator of TGFβ and myostatin signalling, it would be expected that this mutant would have enhanced myogenesis; however, primary myoblasts taken from this mutant mouse did not differentiate efficiently in culture, suggesting that SMAD3 has a pro-myogenic role and that SMAD2 is not able to compensate for this role. These results are in accordance with our own data showing a pro-myogenic role for retinoic acid-induced SMAD3.

5.6 SMAD2 regulates KLF4 and myoblast fusion

In addition to a pro-myogenic role for SMAD3, we find that SMAD2 is required for the KLF4-mediated upregulation of *Npnt* and effective myogenic fusion. It is important to note that while myogenic fusion was not completely abolished in cells lacking *Smad2*, myotubes that did fuse were significantly smaller with a higher percentage of mononucleated myocytes. We have shown that *in vivo* loss of *Smad2* leads to decreased muscle cross-sectional area with no change in total muscle fibers numbers and impaired regeneration after acute injury. It is evident in the representative images taken from newly weaned *Smad2*^{cKO} muscle sections that the fiber numbers are increased compared to WT controls, suggesting that while muscle develops normally, nascent myoblasts are unable to fuse efficiently. Indeed, given that the percentage of PAX7 positive cells was unchanged between WT and *Smad2*^{cKO} muscle sections, we can conclude that the smaller muscle calibre observed in *Smad2*^{cKO} animals is not due to a loss of satellite cells.

Deleting C-terminal serines responsible for activation by TGFβ using a mutant SMAD2ΔSSMS stable cell line showed that this mutant still retained the ability to increase fusion, which would suggest these pro-fusogenic actions are independent of classical TGFβ signalling. Even in the absence of classical C-terminal phosphorylation, SMAD2 could still enter and remain in the nucleus to affect transcription, though it remains unclear how SMAD2 is exerting its action in myoblasts. It is also possible that SMAD2 may have non-canonical actions, independent of TGFβ while being in the cytoplasm that induces its pro-fusogenic function. Indeed, recent studies have found evidence for novel, receptor-independent, biological roles for cytoplasmic SMAD2 and SMAD5 (Fang et al. 2017; Kumar et al. 2016). In human embryonic stem cells, SMAD5 nucleocytoplasmic location was influenced by physiological pH range. Increases in pH did not influence phosphorylation, rather it dissociated charged protons from

specific amino acids in SMAD5's MH1 domain, resulting in accumulation of SMAD5 in the cytoplasm (Fang et al. 2017). This cytoplasmic re-location resulted in the interaction of SMAD5 with hexokinase 1 (HK1) and regulation of glycolysis, independent from the BMP-mediated C-terminal serine phosphorylation, and SMAD4 (Fang et al. 2017). Interestingly, only a SMAD5 basic domain mutant conferring largely cytoplasmic localization was able to rescue mitochondrial dysfunctions seen in SMAD5 knockout cells. Another example of "inactive" cytoplasmic SMAD2 function was described by Kumar and colleagues. This group found that cytoplasmic SMAD2 (and not SMAD3) acted as a scaffold protein and promoted the specific interaction of RIN1 (Rab and Ras interactor-1) with mitofusin-2 (Mfn2) for its activation and was required for the positive regulation of mitochondrial fusion (Kumar et al. 2016). This SMAD2 function was found only in the absence of C-terminal phosphorylation as TGFβ treatment dissociated the SMAD2-Mfn2-RIN1 protein complex and inhibited mitofusion. It remains to be established whether in myocytes, cytoplasmic SMAD2 interacts with mitofusins to enhance mitochondrial fusion. Interestingly, treatment of C2C12 cells with the compound resveratrol enhanced mitochondrial fusion through mitofusin-2, while another study found resveratrol suppressed SMAD2/3 phosphorylation (Robb et al. 2017; C.-L. Chen et al. 2017). These results are interesting in light of our own findings in that for the first time, non-canonical cytoplasmic actions were shown for SMAD proteins which are mainly thought to be inactive in the cytoplasm.

Given that SMAD motifs were found in close proximity and sometimes overlapping with KLF4 motifs, it is possible that KLF4 or other transcription factors act to anchor or stabilize SMAD2 on the DNA to act on its target genes. Perhaps linker phosphorylation could enhance SMAD2 import or inhibit SMAD2 export into the nucleus as well as increase DNA binding.

Future directions would include performing a mass-spectrometry experiment in C2C12 cells using constructs expressing full length SMAD2 and a mutant SMAD2ΔSSMS in the presence or absence of TGFβ treatment. While phosphorylation and acetylation are the most common SMAD2/3 post-translational modifications, these and other candidate post-translational modifications that are differentially modified with TGFβ treatment in either full length or mutant SMAD2 could be further investigated by creating modification-dead or constitutively active modification mutants and evaluate myogenic differentiation and fusion.

5.7 Forced expression of SMAD2 enhances differentiation and fusion

We observed a potent increase in both fusion and differentiation in cells overexpressing SMAD2, a phenotype opposite from which was seen in cells lacking SMAD2, where myotube size was decreased. These results suggest a positive role for SMAD2 in regulating terminal myoblast differentiation and fusion. Proteins identified specifically in the process of fusion include TMEM8C (myomaker), CDK16, IL-4 and the KLF family of transcription factors, KLF2 and KLF4 (Sunadome et al. 2011; Horsley et al. 2003; Millay et al. 2013). KLF2 and KLF4 have been shown to regulate C2C12 myoblast fusion as part of downstream effectors of the ERK5 pathway, without influencing the differentiation program (Sunadome et al. 2011). We observed decreased KLF4 levels in cells lacking SMAD2, consistent with the phenotype of impaired fusion, and using ChIP and luciferase assays, identify *Klf4* as a SMAD2 target gene during myoblast fusion. We have also found that forced expression of KLF4 enhances fusion and stimulates *Npnt* (nephronectin) mRNA levels. Overexpression of KLF4 in cells lacking *Smad2* was unable to stimulate fusion or expression of *Npnt*. These results suggest that *Smad2* is required for expression of *Npnt* and optimal myogenic fusion. Although myomaker was shown to be a potent fusogenic protein, little is known about its regulation and we were unable to correlate

its expression with increased fusion observed in our system, for example by forced expression of *Klf4*, which created large sheets of muscle.

Interestingly, single site analysis of genes involved in the myogenic fusion process revealed multiple KLF4 and SMAD binding sites at potential regulatory regions, using search parameters of -/+ 1 kb relative to the gene's transcription start site. Indeed, we found multiple KLF4 and SMAD binding sites -/+ 1 kb of the *Anxa1* (Annexin A5), *Cdk15*, *Cxcr4*, *Il-4*, *Nfatc2* and *Wasl* genes, with an average of 50% of these regions overlapping or within 20 bp of each other. No binding sites for KLF4 or SMAD were found in proximity to the *Tmem8c* gene using the same parameters, although we did find KLF4, SMAD and NFATC2 motifs further in the -2 kb region which were within 50 bp of each other. The most striking number of KLF4 and SMAD binding sites were observed for *Cdk15* and *Nfatc2* genes, with 71% (5 SMAD and 7 KLF4 sites) and 78% (26 SMAD and 33 KLF4 sites) of binding regions being at close proximity to each other, respectively. It remains to be assessed whether KLF4 and SMAD actually co-occupy these regions, have transcriptional activity alone or cooperate to synergistically activate these fusogenic genes.

KLF4 family members have been shown to form complexes with SMAD proteins to activate transcription of their targets in various contexts. For example, KLF4 was shown to physically interact with SMAD3's MH2 domain to inhibit its activity on the α-SMA promoter (Hu et al. 2007). Given the known collaboration of SMAD3 with master transcription factors such as MYOD and OCT4 and our findings that SMAD2 and KLF4 occupy sites at close proximity of the *Npnt* promoter, it remains possible that transcription factors such as KLF4 could also recruit SMAD2 to promote regulation of their own target genes. For example, it is known that SMAD2 is essential for the maintenance of pluripotency in human and mouse embryonic stem cells as a

direct regulator of *Nanog* expression and suppressor of BMP signalling (Sakaki-Yumoto et al. 2013). It is possible that KLF4, a known transcription factor important for pluripotency, directs SMAD2 to its target genes in embryonic stem cells and also for other cellular actions, such as fusion, in other cell types and in different contexts. Given that SMAD2 occupies the *Klf4* promoter and can activate *Klf4* transcription, that KLF4 drives *Npnt* expression, and both SMAD2 and KLF4 bind the *Npnt* promoter and -12 kb region, it would be interesting to investigate *Npnt* promoter activity using a reporter assay in the presence of both SMAD2 and KLF4 to determine if these proteins function cooperatively. Indeed, in vascular smooth muscle cells, KLF4 and SMAD2 were found to cooperatively activate the TGFβ-Receptor-1 by creating a stable KLF4-SMAD2 complex on SMAD binding regions on the DNA (H. Li et al. 2010). Therefore it remains possible that KLF4 forms a stable complex with SMAD2 on the *Npnt* promoter and cooperate to activate *Npnt* transcription in muscle cells.

Since SMAD affinity for DNA is weak, especially for SMAD2, it is believed that R-SMADs use DNA-binding co-factors to elicit their response upon TGFβ signals. To add to the complexity, these co-factors, whether activating or repressing, could be shared in all cell types while others may be only dependent on the cell type and context. As we are interested in uncovering transcriptional networks controlled by SMAD2 during terminal differentiation and fusion, it would be valuable to perform an RNA-sequencing experiment using vehicle (WT) and tamoxifen-treated *Smad2*$^{fl/flCreER}$ (*Smad2*^{cKO}) stable cell lines and primary myoblasts overexpressing SMAD2 and empty vector controls could be performed. Cell samples should include proliferating myoblasts in growth conditions and myoblasts differentiated for 6 hours and after 2 days, time points for genes involved in early and late fusion events. Genes differentially upregulated and downregulated (1.5-fold difference) in cells lacking or overexpressing *Smad2*

WT in growth conditions versus low-serum conditions will be further investigated. Using gene ontology analysis, we will focus on genes related to fusion events including but not limited to: migration, cell adhesion and cytoskeletal assembly and interesting candidates will be validated by RT-qPCR before performing loss and gain of function and rescue experiments using the validated targets. An important future direction of this work also includes identifying SMAD2 binding co-factors and their specific binding motifs in myoblasts. To this end, ChIP for SMAD2 in myoblasts will be performed, followed by de-crosslinking and mass-spectrometry to identify proteins that were bound to SMAD2 on the chromatin. Alternatively, proximity-dependent biotin identification (BioID) experiments followed by mass-spectrometry could help identify SMAD2 binding co-factors in the nucleus in unstimulated cells. This technique has been shown to be helpful particularly in mapping protein-protein interactions that are not direct but could have biological significance as well as weak interactions.

5.8 TGFβ-independent SMAD transcriptional activity

As mentioned above, it seems more and more likely that SMAD proteins possess non-canonical cytoplasmic functions in their unphosphorylated form and could have more TGFβ-independent transcriptional activity than previously thought. For example, expression of SMAD7, a specific inhibitor of SMAD2 and SMAD3, could not rescue the TGFβ-induced inhibition of myogenesis, but did rescue myostatin-induced block in myogenesis (Kollias et al. 2006). Thus, SMAD-independent TGFβ mechanisms that contribute to inhibit myogenic differentiation must exist. We hypothesize that one of these non-canonical TGFβ mechanisms could be the upregulation of C/EBPβ expression, observed after treatment of myoblasts with TGFβ.

5.9 SMAD nuclear import

The difference in structure, particularly in the MH1 domain, between SMAD2 and SMAD3 means that their nuclear import mechanism differs and could regulate differential transcriptional activity. Phosphorylated SMAD3 uses importin β1 through interaction with its MH1 domain, while SMAD2 cannot due the presence of exon 3 which disrupts the nuclear localization signal (Kurisaki et al. 2001). Instead, SMAD2 contacts the nuclear pore complex directly by making contact with specific nucleoporins via its MH2 domain, even in the absence of C-terminal phosphorylation (Xu et al. 2002). SMAD3 also has a nuclear export signal (NES) located in the MH2 domain, which is recognized by exportin-4 for export into the cytoplasm (Kurisaki et al. 2006; Zhan Xiao et al. 2003). A canonical NES has not been found for SMAD2, and this could explain why it does not interact with exportins. It is also known that RanBP3 exports unphosphorylated SMAD2 and SMAD3; a phosphorylation event other than one caused by TGFβ receptors, could inhibit this interaction and decrease the export rate of SMAD2 and SMAD3 (Dai et al. 2009).

In unstimulated cells, SMAD2 enters the nucleus but is rapidly exported, resulting in greater cytoplasmic localization in the absence of TGFβ. It was found that TGFβ treatment does not affect the import rate of SMADs, rather it decreases their export rate out of the nucleus (Schmierer and Hill 2005). A nuclear phosphorylation event or another post-translational modification could modulate the affinity of SMAD2 for the export machinery, and thereby increase its nuclear presence. Similarly, it is possible that a yet unknown protein kinase could activate SMAD2 in the cytoplasm at its linker region for example, and lead to a pool of activated SMAD2 in the nucleus. Indeed, the MEKK-1 kinase, involved in the ERK/JNK pathway, was found to activate SMAD2 by an independent phosphorylation event not requiring classical SSXS

motif phosphorylation by the TGFβ receptor (Brown et al. 1999). SMAD2 activation by MEKK-1 induced nuclear accumulation in the absence of TGFβ and enhanced the formation of SMAD2:co-activator complexes on target promoters in endothelial cells. Interestingly, forced MEKK-1 expression in C2C12 myoblasts cells induced myogenin expression (Cabane et al. 2003). It would be interesting to speculate that MEKK-1 could induce activation of SMAD2 and perhaps this phosphorylation event would stabilize a SMAD2:KLF4 complex on the myogenin promoter, inducing *Myog* expression. Preliminary data from our lab shows that KLF4 could activate the myogenin promoter. It would be interesting to know whether KLF4 could still induce myogenin expression in a SMAD2-null background, as SMAD2 was required for the activation of *Npnt* by KLF4. Similarly, in unstimulated cells, ERK1 can phosphorylate SMAD2 and cause an increase in total SMAD2 protein (Funaba, Zimmerman, and Mathews 2002). The amino acid in question, Thr8 was phosphorylated independently of TGFβ by ERK1 and lead to increased SMAD2 stability and enhanced transcriptional activity (Funaba, Zimmerman, and Mathews 2002). These results suggest that post-translational modifications independent of TGFβ activation can induce SMAD2 nuclear accumulation and transcriptional activity.

5.10 Conclusions

The objectives of this research project were to investigate the roles of the transcription factors SMAD2 and SMAD3 during myogenic differentiation. Although SMAD3 is known as an effector protein for canonical TGFβ signalling, which is potently anti-myogenic, we have identified a pro-myogenic role for SMAD3 through its activation by RA and subsequent disruption of C/EBPβ transcriptional activity. We found that TGFβ increased C/EBPβ expression in myoblasts, which could be reversed by RA treatment. Further, primary myoblasts lacking *Cebpb* were less sensitive to TGFβ treatment. In the second part of this study, we developed a

novel mouse model in order to study the effect of loss of *Smad2* on muscle. Using this model where *Smad2* is excised in PAX7-expressing satellite cells upon treatment with tamoxifen, we characterized the differentiation and fusion as well as the regeneration phenotype upon injury and found a positive role for SMAD2 in regulating myogenic fusion. We found that SMAD2 regulates expression and activity of *Klf4* early in differentiation and is required for the *Klf4*-induced expression of *Npnt* and productive fusion. We have therefore identified a novel regulatory pathway for myogenic fusion involving the regulation of *Klf4* by SMAD2 and the requirement for SMAD2 in KLF4-mediated increase in *Npnt*. This work brings more knowledge into the intricacies of the TGFβ signalling pathway in muscle cells. However, given the newly discovered role for unphosphorylated cytoplasmic SMAD2 and SMAD5, we believe there is much more to be discovered for SMADs and their role independently of TGFβ signalling. Further, cytoplasmic SMADs might be used as extra sensitive sensors for determining changes in the cellular environment and to fine-tune cellular responses, whether transcriptionally or non-transcriptionally. As we highlight a role for SMAD2 in terminal myogenic differentiation and fusion, it is important to keep in mind the broad actions of transcription factors, including SMAD proteins, when targeting a signalling pathway as a therapeutic strategy.

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