

ROLE OF A SET/Smad7 INTERACTION IN SKELETAL MYOGENESIS

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Abstract

Skeletal muscle differentiation (myogenesis) is the process of converting mono-nucleated myoblast cells into multinucleated differentiated myotubes that form the basis of the skeletal musculature. Myogenic regulatory factors (MRFs) are sequence specific, DNA binding transcription factors that play a central role in myogenesis. Two of the MRFs, MyoD and Myogenin, are essential to the initiation of the irreversible differentiation program. TGF β is the prototypic member of the TGF β super-family of cytokines and a potent inhibitor of myogenesis. Inhibitory Smads (I-Smad) repress receptor regulated Smads (R-Smads) activation by TGF β and exogenous expression of Smad7 (one of the two I-Smads) enhances myogenesis as indicated by premature expression of myogenic marker genes such as *myogenin*, *mck*, and *myhc*.

Previously, our group showed that exogenously expressed nuclear Smad7 was able to induce early myogenesis, indicating that Smad7 has a role in the nucleus, separate from its cytoplasmic function and this role contributes to its enhancing properties on myogenesis. A recent screen for Smad7 interacting proteins indicated an interaction between Smad7 and SET/TAF-I β , a nuclear protein which plays diverse functions in chromatin remodeling, cell cycle, autophagy, and apoptosis. The focus of this study was to characterize the roles of Smad7 and SET, and the interaction of the two impacts myogenesis. Here we report that ectopic expression of SET inhibits myogenic differentiation. However, co-expression of SET and Smad7 resulted in a strong enhancement of myogenesis, leading to the formation of more robust myotubes and higher levels of myogenic markers. Using ChIP-qPCR, we quantified levels of

activating and repressive histone marks on regulatory regions of several muscle specific genes such as *myogenin* and *muscle creatine kinase (mck)*.

These data indicate that expression of exogenous SET alone led to higher levels of transcriptionally repressive marks (H3K27me3) and lower levels of activating marks (H3K4me3/H3K9Ac) on muscle specific regulatory regions. Contrary to the effects seen with SET alone, expression of exogenous Smad7 resulted in higher levels of transcriptionally activating marks (H3K4me3/H3K9Ac) and lower levels of the transcriptionally repressive mark (H3K27me3) compared to that of control. Interestingly, co-expression of exogenous SET and Smad7 led to higher levels of transcriptionally activating marks (H3K4me3/H3K9Ac) while also exhibiting lower levels of the transcriptionally repressive mark (H3K27me3) compared to that of Smad7 alone.

Taken together these data indicate that SET can function as an inhibitor of transcription for myogenic related genes, while the interaction with Smad7 relieves, and enhances transcription of those myogenic genes. In contrast to the “myogenic” genes, exogenous Smad7 had no effect on SET’s capability to repress the *cyclin D1* promoter. This may explain the enhancement of myogenesis with SET and Smad7 together since cell cycle withdrawal, which is mediated by *cyclin D1* suppression, is a prerequisite of differentiation. Therefore, SET mediated repression of cell cycle coupled with Smad7’s antagonism of SET at myogenic genes results in a pronounced enhancement of myogenesis. These observations further explain the role of Smad7 in the molecular control of myogenesis, having wider implications for understanding skeletal muscle physiology and pathology.

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Table of Contents

Abstract.....	ii
Acknowledgements	iv
Table of Contents	v
List of Figures.....	vii
List of Abbreviations	viii
Review of Literature	1
Origin of skeletal muscle	1
Molecular biology of skeletal muscle development	3
Pax3 in skeletal muscle development	3
Pax7 in skeletal muscle development	4
Specification of skeletal muscle progenitors	4
Myogenic regulatory factors (MRFs) in skeletal muscle development	6
MEF2 transcription factors in skeletal muscle development.....	8
The Transforming Growth Factor β (TGF β) Signaling pathway.....	9
TGF β ligand processing and receptor interaction	11
The ‘Canonical’ TGF β signalling pathway through R-Smads	12
Inhibitory I-Smads	14
Smad6 in TGF β signaling	15
Smad7 in TGF β signaling	15
Smad independent, ‘noncanonical’ TGF β Signalling pathways.....	17
Smad7 regulation	17
Smad7 sub-cellular localization.....	18

Nuclear function of Smad7	19
Pathophysiological roles of Smad7	20
Role of TGF- β signaling in myogenesis	21
Role of Smad7 in myogenesis.....	22
Epigenetic changes during skeletal muscle differentiation.....	24
Statement of Purpose	26
Introduction.....	27
Materials and Methods.....	29
Results	33
Discussion.....	51
Summary and Future Direction	58
References	61
Supplementary Figures	72
Supplementary Tables	75
Extended Materials and Methods.....	76

List of Figures

Figure 1: Schematic diagram showing the origin of skeletal muscle in the developing embryo and the different stages of skeletal muscle differentiation.

Figure 2: Schematic diagram of major cell-cell communications at the dorsal medial lip in the developing embryo.

Figure 3: Schematic diagram of TGF β ligand/receptor combinations.

Figure 4: Schematic diagram TGF β ligand processing and activation

Figure 5: Schematic diagram of different domains in Smad proteins.

Figure 6: Schematic Diagram of Canonical TGF β signalling through Smad proteins

Figure 7: Schematic diagram of Smad7 nuclear function in skeletal muscle differentiation

Figure 8: Exogenous SET and Smad7 interact in HEK293T cells.

Figure 9: Exogenous SET and Smad7 promote myogenesis while ectopic expression of SET alone leads to cell death.

Figure 10: C2C12 differentiation timecourse with exogenous Smad7 and SET.

Figure 11: Luciferase assays of MyoG and MCK in the presence of exogenous SET and/or Smad7.

Figure 12: Endogenous mRNA levels of SET and Smad7 in a C2C12 differentiation timecourse.

Figure 13: Myogenic marker mRNA levels in a C2C12 differentiation timecourse in the presence of exogenous SET and/or Smad7.

Figure 14: Modulation of total levels of histone marks H3K4me3, H3K9Ac, and H3K27me3 in the presence of exogenous SET and/or Smad7 in a C2C12 differentiation timecourse.

Figure 15: Epigenetic changes to the regulatory regions of *mck*, *myogenin*, *cyclin D1*, and *gapdh* in the presence of exogenous SET and/or Smad7.

Figure 16: Comparison of the structure and functional domains of SET and NAP1. The NAP domain is required for histone protein binding while the highly acidic C terminal domain is required for binding and forming the INHAT complex.

Figure 17: Schematic diagram summarizing the epigenetic changes to the regulatory regions of *mck*, *myogenin*, *cyclin D1*, and *gapdh* in the presence of exogenous SET and/or Smad7.

Figure 18: Epigenetic changes to muscle specific genes and the *cyclin D1* gene when introducing ectopic SET and Smad7.

List of Abbreviations

AICD – Alzheimer Disease Amyloid Protein
AIP4 – Itchy E3 Ubiquitin Protein Ligase
AP-1 – Activator Protein-1
APE-1 – Apurinic/aprimidinic 1
ATF3 – Cyclic AMP-dependent Transcription Factor
bHLH – basic Helix-Loop-Helix
BMP – Bone Morphogenetic Protein
BMPR – Bone Morphogenetic Protein Receptor
BRG1 – SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin, Subfamily A
CBP – CREB binding protein/p300
CDK – Cyclin Dependent Kinase
ChIP – Chromatin Immuno-Precipitation
COUP-TF – Nuclear Receptor Subfamily 2, group F
CREB – cAMP Response Element-Binding Protein
CRM-1 – Chromosome Region Maintenance 1
DHEA – Dehydroepiandrosterone
DML – Dorsal Medial Lip
ECM – Extracellular Matrix
EGF – Epidermal Growth Factor
ERK – Extracellular signal-Regulated Kinase
Ezh2 – Enhancer of Zeste Homolog 2
Fe65 – Amyloid Beta Precursor Protein Binding Family B Member 1
FGF – Fibroblast Growth Factor
Furin – Paired Basic Amino Acid Cleaving Enzyme
GADD34 – Growth Arrest and DNA Damage-34
GCN5 – Lysine Acetyltransferase 2A
GDF – Growth Differentiation Factor
GRE – Glucocorticoid Response Elements
H3K27me3 – trimethylated Lysine 27 on Histone 3
H3K4me3 – trimethylated Lysine 4 on Histone 3
H3K9Ac – Acetylated Lysine 27 on Histone 3
HAT – Histone Acetyltransferase
HDAC – Histone Deacetylase
I2PP2a – Phosphatase 2A Inhibitor
IBD – Inflammatory Bowel Disease
IGAAD – Inhibitor of Granzyme A-Activated DNase
IGF1 – Insulin-like Growth Factor-1
ING1 – Inhibitor of Growth 1
INHAT – Inhibitor of Histone Acetylation
JNK – Jun N-terminal Kinase
KAI1 – Kangai 1 / CD82 Molecule
KDM6A – Lysine Demethylase 6A
LAP – Latency Associated Peptide

LLC – Large Latent Complex
LTBP – Latent TGF β -Binding Protein
MAPK – Mitogen-Activated Protein Kinase
MB – Myoblast
MCK – Muscle Creatine Kinase
MDC1A – Laminin α 2-deficient congenital muscular dystrophy type 1A
MEF2 – Myocyte Enhancer Factor-2
MH1 – MAD (Mothers against decapentaplegic) Homology-1
MH2 – MAD (Mothers against decapentaplegic) Homology-2
MKK – Mitogen-Activated Protein Kinase Kinase
MLL1 – Mixed-Lineage Leukemia 1
MMP – Matrix metalloproteinases
MRF4 – Muscle Regulatory Factor 4
MT – Myotube
MVG – Mean Grey Value
Myf5 – Myogenic Factor-5
MyHC – Myosin Heavy Chain
MyoD – Myogenic Differentiation 1
MyoG – Myogenin
NAP1 – Nuclear Assembly Protein 1
NEDD4-2 – Neural precursor cell Expressed Developmentally Down-regulated protein 4
NES – Nuclear Export Signal
NF-K β – Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NGF-IB – Nerve Growth Factor IB
NLS – Nuclear Localization Signal
P/CAF – P300/CBP associated factor
p38MAPK – p38 Mitogen Activated Protein Kinase
P450c17 – Steroid 17-alpha-monooxygenase
Pai1 – Plasminogen Activator Inhibitor-1
Pax3 – Paired box gene-3
Pax7 – Paired box gene-7
PK1 – Pyruvate dehydrogenase Kinase 1
PI3K – Phosphatidylinositol 3-Kinase
PP2A – Serine/threonine Protein Phosphatase 2A
PRC2 – Polycomb Repressive Complex 2
PTM – Post Translational Modification
RT-qPCR – Reverse Transcriptase quantitative Polymerase Chain Reaction
SAD – Smad4 Activation Domain
SBE – Smad Binding Element
SET – SET proto-oncogene
Setd7 – SET Domain Containing Lysine Methyltransferase 7
SF-1 – Steroidogenic Factor 1
Shh – Sonic hedgehog
SIRT1 – Sirtuin (silent mating type information regulation-2) homolog-1
SIS3 – Smad3 specific inhibitor
SLC – Small Latent Complex

Smad – Mothers Against Decapentaplegic
Smurf – Smad ubiquitin Regulatory Factor
Sp1 – Trans-acting Transcription Factor 1
STAT – Signal Transducer and Activator of Transcription
STRAP – Serine/Threonine kinase Receptor Associated Protein
SWI/SNF – SWItch/Sucrose Non-Fermentable
TAF-I β - Template-Activating Factor I β
TAK1 – Transforming growth factor beta-activated kinase 1
TAZ – Transcriptional co-activator with PDZ-binding motif
TFE3 – Transcription Factor Binding To IGHM Enhancer 3
TGF β – Transforming Growth Factor β
TGF β R – Transforming Growth Factor β Receptor
Tip60 – Lysine Acetyltransferase 5
VLL – ventral lateral lip
Wnt – Wingless-type MMTV integration site family member
Yy1 - Yin and Yang 1

Review of Literature

Origin of skeletal muscle

Skeletal muscle is an essential tissue that allows locomotion and supports the proper posture for vertebrates. With the exception of some facial and oesophageal muscles, all skeletal muscle fibres of the body come from progenitor cells that originate from somites. Somites are transient spheres of cells that originate from the paraxial mesoderm, forming on either side of the neural tube during ontogeny.

These newly formed epithelial cells are divided further into distinguishable groups of cells such as the sclerotome, syndetome, dermomyotome, and the myotome. (**Figure 1B**) The syndetome gives rise to tendons while the sclerotome gives rise to bones and cartilage. The dermomyotome gives rise to dermis and to the progenitor cells of the myotome and the cells at the epaxial myotome give rise to the deep back muscles while the hypaxial dermomyotome provides muscle precursor cells that migrate and form the skeletal musculature of the limbs and the lateral trunk muscles (Brent et al., 2003, Brent and Tabin, 2002, Buckingham et al., 2003).

The progenitor cells in the ventral lateral lip delaminate from the dermomyotome, migrate into the anatomical positions that muscle fibres must form on the limbs, where they proliferate, express myogenic determination factors and subsequently differentiate into myotubes (**Figure 1A**) (Ordahl and Le Douarin, 1992, Marcelle et al., 1997).

The differentiated myotubes are multinucleated cells expressing muscle specific gene markers such as *muscle creatine kinase (mck)* and *myosin heavy chain (myhc)* which contribute to the final stage of muscle development, the maturation of myotubes into muscle fibres.

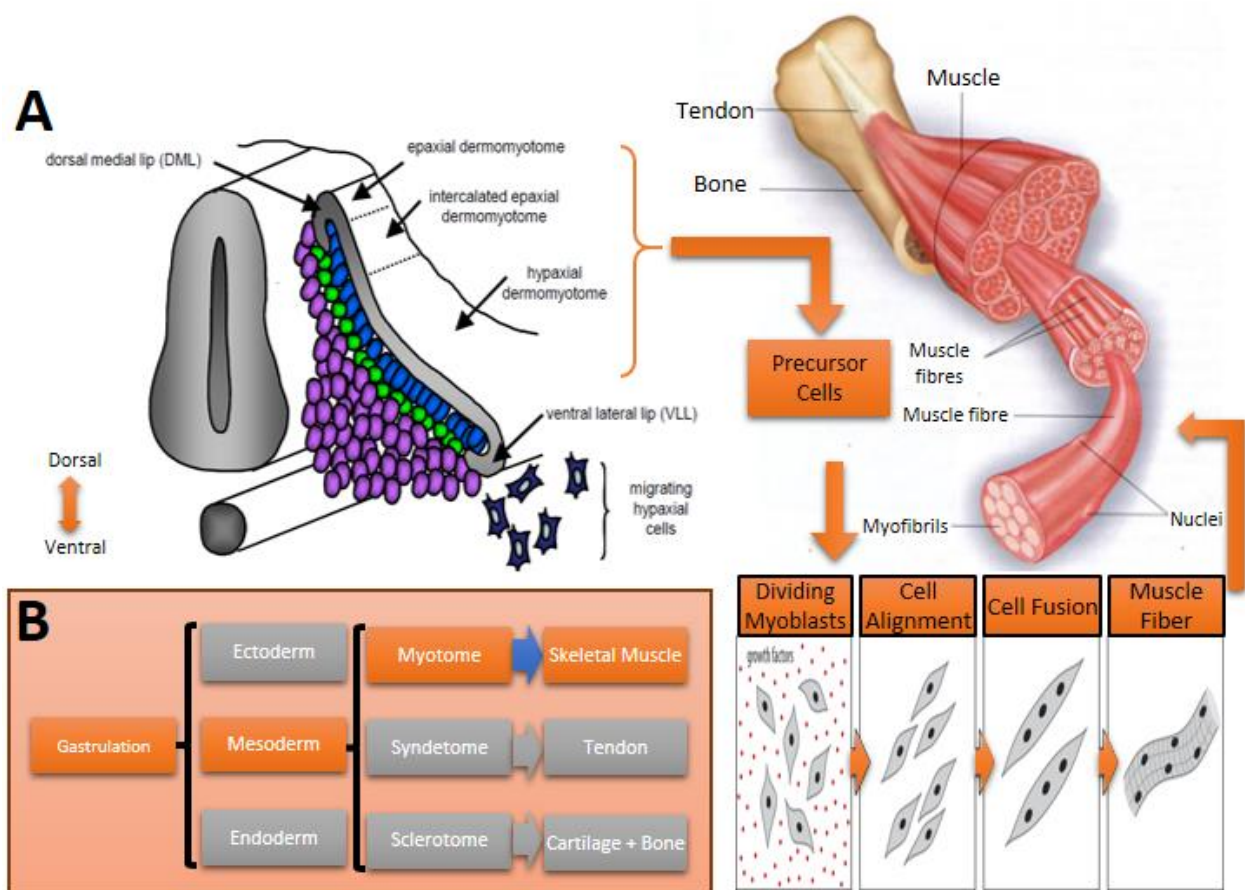


Figure 1: Schematic diagram showing the origin of skeletal muscle in the developing embryo and the different stages of skeletal muscle differentiation. Images adapted from (Staveley, 2010, Miyake, 2009, Sindhu, 2014)

Molecular biology of skeletal muscle development

Pax3 in skeletal muscle specification

The paired box gene family is characterized by the presence of a paired domain. The Paired box gene-3 (Pax3) is widely expressed in the presomitic mesoderm and is also found in newly formed epithelial somites. Pax3 is the first known protein that is involved in specification of the skeletal muscle lineage (Chi and Epstein, 2002, Marcelle et al., 1997, Lang et al., 2007). Exogenous *in vitro* expression of Pax3 in the lateral mesoderm induces expression of both myogenic differentiation 1 (MyoD) and myogenic factor-5 (Myf5), two downstream transcription factors that play major roles in the commitment and differentiation of muscle cells (Marcelle et al., 1997).

Embryos lacking the Pax3 protein (Pax3^{-/-}) don't form limb muscles, suggesting that Pax3 is required for the migration of the precursor cells from the ventral lateral lip (VLL) of the hypaxial dermomyotome to the limbs (Goulding et al., 1994). Furthermore, embryos lacking Pax3 and Myf5 proteins fail to form muscle anywhere except some head muscles. This suggests that Pax3 regulates the initiation of the myogenic gene expression program since the *myf5* gene is also regulated by Pax3 (Tajbakhsh et al., 1997). Indeed, exogenous expression of Pax3 was sufficient enough to convert mesoangioblasts and P19 embryonic carcinoma cells into the skeletal muscle lineage (Ridgeway and Skerjanc, 2001).

It is important to note that Pax3 expression is only important to initiate the myogenic gene expression program as Pax3 expression has not yet been detected in adult skeletal muscles, with the exception of a small fraction of musculatures and satellite cells (Lepper et al., 2009). In fact, exogenous Pax3 inhibits the normal course of myogenesis in C2C12 cells (Epstein et al., 1995). Even in satellite cells, Pax3 is only transiently upregulated upon activation and

proliferation, suggesting that Pax3 may only be required for activation and early proliferation, but Pax3 levels need to be downregulated for progression of myoblast differentiation (Boutet et al., 2007).

Pax7 in skeletal muscle development

Another essential member of the Pax gene family is Pax7, and the canonical view is that Pax7 is a major player in specification and survival of satellite cells (Seale et al., 2000). Pax7 was found to be expressed in the central region of the dermomyotome (**Figure 2**), and has been shown to compensate some of Pax3's role in the dorsal neural tube, neural crest cell, and somite development (Mansouri et al., 1996). However, loss of Pax7 led to complete loss of satellite cells resulting in regeneration defects in the postnatal mouse (Seale et al., 2000). Loss of both Pax3 and Pax7 causes complete arrest in skeletal muscle development after the primitive myotome is formed (Relaix et al., 2005). It is evident that Pax7 is essential for satellite cell survival and Pax3 is required for migration of muscle progenitor cells to the limb and commitment to the myogenic lineage.

Specification of skeletal muscle progenitors

The newly formed epithelial somites are divided into pools of progenitor cells, with the myotome giving rise to the skeletal muscle in the body. Specification of the myotome is highly dependent on the position of the cells in the newly formed somite, and communication with the surrounding axial structures by means of extracellular protein-protein interactions or soluble secreted factors are required for commitment into the myotome progenitor pool (Marcelle et al., 1997).

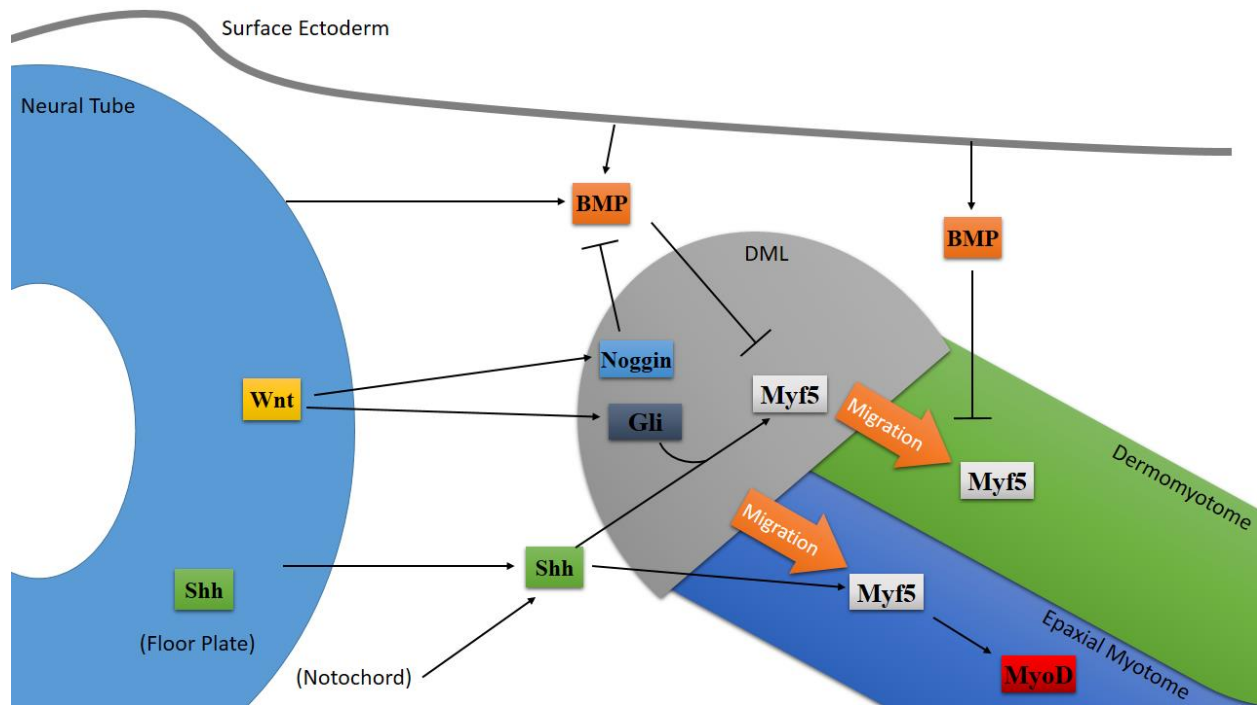


Figure 2: Major cell-cell communications at the dorsal medial lip (DML) in the developing embryo. Adapted from (Miyake, 2009).

One of the key players in specification of the myotome is the sonic hedgehog (Shh) protein, which is secreted from the notochord and the floor plate of the neural tube and is required for activating the transcription factor Myf5 in the dorsal medial region (DML) and epaxial myotome. Wnt signalling from the neural tube also contributes to the specification of DML by activating the Noggin and Gli transcription factors that further activate Myf5. Conversely, the neural tube secretes Bone Morphogenetic Proteins (BMPs) which inhibit Myf5 expression in the hypaxial myotome. However, since Noggin, an antagonist of BMP signalling is present due to Wnt signalling, BMP signalling is unable to inhibit Myf5 in the DML (Fan et al., 1995, Gustafsson et al., 2002, Johnson et al., 1994, Münsterberg et al., 1995).

The high BMP levels from the dorsal ectoderm keep the hypaxial muscle precursors in an undifferentiated state due to BMPs ability to successfully inhibit Myf5 expression in hypaxial

muscle progenitors. However, since Wnt signaling induces both Gli (agonist of Myf5), and Noggin (antagonist of BMP), *myf5* gene expression is still induced in the DML (Cossu et al., 1996, Pourquié et al., 1995, Pourquié et al., 1996, Schnapp et al., 2009, Sporle et al., 1996, Takada et al., 1994).

The ventral region of the somites also have Myf5 since the Shh secretion from the notochord and floor plate of the neural tube induce Myf5 expression. Myf5 presence in these progenitor cells results in expression of MyoD, a major skeletal muscle transcription factor that guides the cells to differentiate into myotubes.

The notochord and the floor plate of the neural tube therefore play a pro-differentiation role in the formation of myocytes and epaxial muscles on the ventral side while the dorsal end of the neural tube along with the dorsal ectoderm keep the progenitor cells in a proliferative and undifferentiated state, contributing to a growing dermomyotome (**Figure 2**) (Cossu et al., 1996).

Myogenic regulatory factors (MRFs) in skeletal muscle development

The four myogenic regulatory factors, Myf5, MyoD, MyoG, and MRF4 are essential to the induction of the skeletal muscle differentiation program (Yokoyama and Asahara, 2011). The four MRFs are part of the basic helix-loop-helix (bHLH) family of transcription factors and are evolutionarily conserved in vertebrates (Pownall et al., 2002). The MRFs have been shown to have the remarkable ability to convert many nonmuscle cell type into muscle which identifies these transcription factors as key regulators of the differentiation of muscle progenitor cells (Davis et al., 1987, Molkenstein and Olson, 1996). Myf5 and MyoD are highly expressed in the proliferating myoblasts while Myogenin and MRF4 are expressed at later stages of differentiation, suggesting that Myf5 and MyoD establish and maintain the muscle progenitor lineages while Myogenin and MRF4 induce expression of muscle contractile genes including

MyoD and Myf5. Most skeletal muscle specific genes have the a short consensus DNA sequence CANNTG, termed E-box, in their regulatory region and to induce promoter activity of the genes, this sequence needs to be bound by ubiquitously expressed E-proteins (E12, E47) and a heterodimer complex of the MRFs (Etzioni et al., 2005, Murre et al., 1989).

The importance of the function of the MRFs for myogenesis is revealed when looking at the gene targeting studies in mice. Loss of MyoD (Rudnicki et al., 1992) or Myf5 (Braun et al., 1992) alone has no effect on specification of the myogenic lineage in embryo development. However, mice lacking both *MyoD* and *myf5* leads to loss of myoblast and muscle fibers prevailing the importance of Myf5 and MyoD in cell lineage specification (Kassar-Duchossoy et al., 2004).

In contrast to mice lacking *MyoD* and *myf5*, the early steps of myogenesis occurs normally in mice lacking *myogenin*. However, secondary myogenesis, which leads to the formation of myofibers, does not take place in *myogenin* mutants, and *myogenin*(-/-) mice die prenatally because of the complete absence of differentiated muscle. Instead of myotubes containing multinucleated myocytes, only mononucleated cells are present in muscle tissue of *myogenin*(-/-) mice (Hasty et al., 1993).

Mutations of *mrf4* result in largely varied phenotypes, from minor rib cage defects in viable mice, to perinatal lethality of mice (Patapoutian et al., 1995, Zhang et al., 1995). Since the MRF4 and Myf5 locus are within 8kb of each other in mice, any mutation on *mrf4* affects the expression of the *myf5* gene leading to phenotypes similar to mutations on *myf5* that downregulate *myf5* expression. (Yoon et al., 1997) As a result, it appears likely that the rib phenotype observed in *mrf4* mutants was not because of the loss of MRF4 function, but caused

by the down regulation of Myf5. The function of MRF4 needs to be elucidated further before any clear, distinct function for MRF4 is established.

MEF2 transcription factors in skeletal muscle development

In mammals, there are four *mef2* genes *mef2a-d*. The MEF2 proteins form homo or heterodimers on dsDNA, on a consensus sequence [(C/T)TA(A/T)₄(TA(G/A))], termed the MEF2 binding site (Pollock and Treisman, 1991). *In vivo* studies revealed that the *mef2* genes are expressed in developing cardiac, skeletal, and smooth muscle cells during embryogenesis but are downregulated after birth, suggesting that the *mef2* genes act synergistically with MRFs to activate muscle specific genes and induce myogenic determination and differentiation. (Black and Olson, 1998, Olson et al., 1995). Indeed, *mef2* genes have been shown to activate several muscle specific genes and when introduced in combination with MRFs, increase efficiency of myogenic conversion of non-muscle cells *in vitro*. *mef2* genes are expressed in a wide variety of tissues after E12.5, suggesting that the activity of MEF2 is post transcriptionally regulated (Ornatsky and McDermott, 1996). MEF2 transcriptional activity was shown to be regulated by phosphorylation and recruitment of either Histone acetyltransferase (HAT) or histone deacetylases (HDACs) (Du et al., 2008).

Most muscle specific genes have, within their regulatory region, both a conserved E-box and MEF2 binding site for MRF and MEF2 members to bind respectively. MEF2 and MRFs have been shown to work together synergistically to upregulate these muscle specific genes in muscle cells, presumably to induce determination and differentiation (Black and Olson, 1998, Olson et al., 1995).

The Transforming Growth Factor β (TGF β) Signaling pathway

One of the key regulators of tissue morphogenesis and embryonic development is the TGF β superfamily, which consists of TGF β s, BMPs, Nodal, Activin, and Growth and differentiation factors (GDFs). Active TGF- β ligand dimers bind to TGF β cell surface receptors, phosphorylating intracellular effector proteins (receptor Smads; or R-Smads), which translocate into the nucleus and regulate target gene expression that leads to a cellular response (Feng and Derynck, 2005, Heldin et al., 1997).

TGF β has been found to have a regulating role in proliferation, migration, adhesion, apoptosis, cell growth and differentiation. For example, TGF β stimulation induces fibrosis and proliferation of fibroblasts (Hocevar et al., 2005), enhances differentiation of neuronal cells (Moon et al., 2009) but blocks differentiation in mesenchymal cells (Bakin et al., 2000). It is apparent that the cellular context in which the TGF β signalling is activated is what dictates the end effect (Feng and Derynck, 2005).

Most cell types express TGF- β family members as they play key roles in differentiation and tissue morphogenesis. Responses to TGF β stimulation are complex due to the high number of possible different ligands and receptor complex formations which depend on the cell context and physiological environment.

There are over 42 ligands from 29 genes that encode the TGF β superfamily ligands that are believed to be secreted out of the synthesizing cell as homo or heterodimers (Shi and Massagué, 2003, Derynck and Zhang, 2003) (**Figure 3**) .

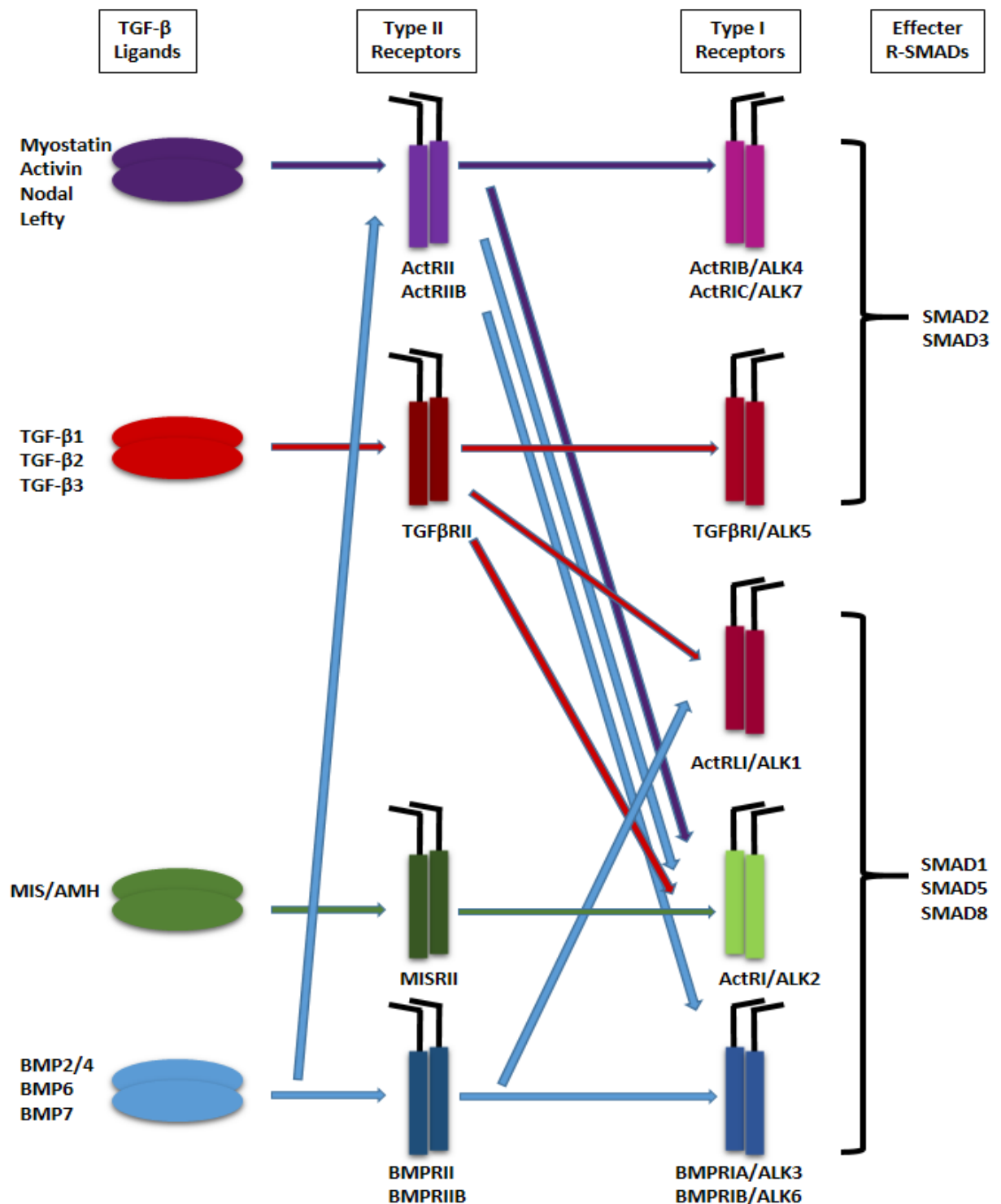


Figure 3: Schematic diagram of TGF β ligand/receptor combinations. Adapted from (Miyake, 2009)

TGFβ ligand processing and receptor interaction

Upon synthesis, the long peptide chain product of the *tgfβ* gene is cleaved via Furin, a proprotein convertase, creating a complex called the Small Latent Complex (SLC) (Derynck et al., 1985, Rifkin, 2005). The SLC and Latent TGFβ-binding protein (LTBP) combine to form the Large Latent Complex (LLC). The LLC is what gets secreted into the extracellular matrix (ECM) (Annes et al., 2003). The Latency Associated Peptide (LAP) and LTBP proteins act as an extracellular trap that isolates TGFβ ligands, preventing any interaction with the TGFβ surface receptors. In order for the ligands to bind to TGFβ surface receptors, the ligands need to be released from the LLC, usually by a secreted enzyme such as Matrix metalloproteinases 2/9 (MMP-2, MMP9) (Yu and Stamenkovic, 2000) or Thrombospondin-1 (Schultz-Cherry and Murphy-Ullrich, 1993). Alternatively, acidic conditions (Lyons et al., 1988) or reactive oxygen species (Barcellos-Hoff and Dix, 1996) can also induce the release of the TGFβ ligands from the LLC and the active TGFβ ligand can then bind to and activate a receptor complex. (**Figure 4**)

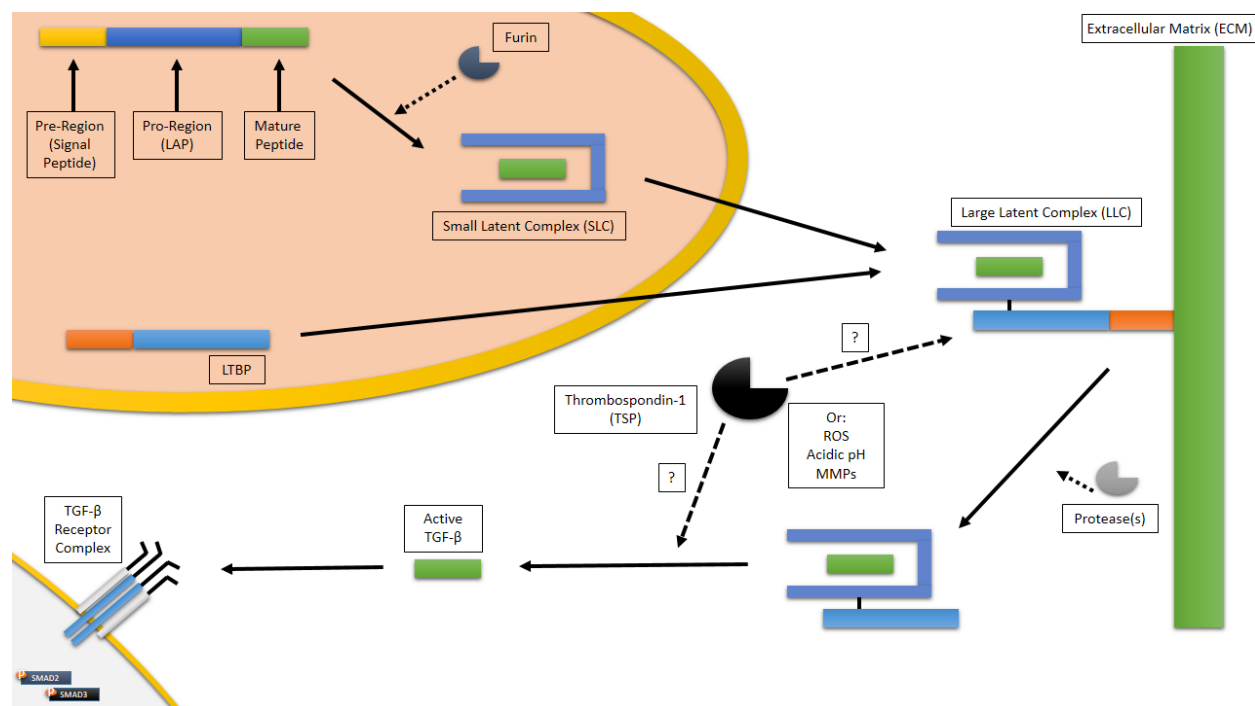


Figure 4: Schematic diagram of TGFβ ligand processing and activation.

The TGF β cell surface receptor complex contains two ‘type I’ and two ‘type II’ transmembrane proteins. The type I & II TGF β receptors have a structurally similar structure with the major difference being a Glycine/Serine-rich ‘GS-rich sequence’ in the cytoplasmic tail of the type I receptor. Without ligand binding, the TGF β receptors form homodimers and are inactive kinases. Once the LLC is cleaved and the active TGF β ligands are released, the ligands are able to bind to the TGF β receptor complex. Binding of the TGF β ligand to the type II receptors stimulates recruitment of two type I receptors, followed by auto-phosphorylation of serine residues in the ‘GS-rich sequence’ of the type I receptor, forming an activated receptor complex (Shi and Massagué, 2003).

So far, seven type I and six type II receptors have been identified, generating a high number of diverse receptor complexes which explains the ligand specificity and diverse biological responses. Some of the ligand/receptor combinations of the TGF β family cytokines are shown in **Figure 3**. In most cases, the TGF β ligands in the canonical TGF β signalling pathway activate R-Smads 2, and -3 while the BMP ligands activate receptor complexes such as BMPRII that recruits R-Smads 1, -5, and -8 (Shi and Massagué, 2003). The cellular response to TGF β stimulation is dictated by the expression of different receptor types by the cell before the TGF β stimulation.

The ‘Canonical’ TGF β signalling pathway through R-Smads

The intracellular signalling molecules of the TGF β ‘canonical’ signalling pathway are the Mothers against decapentaplegic (Smad) proteins. The Smad proteins are functionally categorized into three groups: the receptor Smads (R-Smad 1, 2, 3, 5, and 8), the Common-Smad (C-Smad, Smad4), and the inhibitory Smads (I-Smads, Smad6 and 7). There is a conserved MH2 (Mad Homology 2) domain in the C-terminus of all the Smads with the L3 loop in the MH2

domain responsible for binding to a specific type I receptor in the activated receptor complex. There is a less conserved MH1 domain in the N-termini of the Smad proteins responsible for interaction with DNA (Macias et al., 2015, Shi and Massagué, 2003) (**Figure 5**).

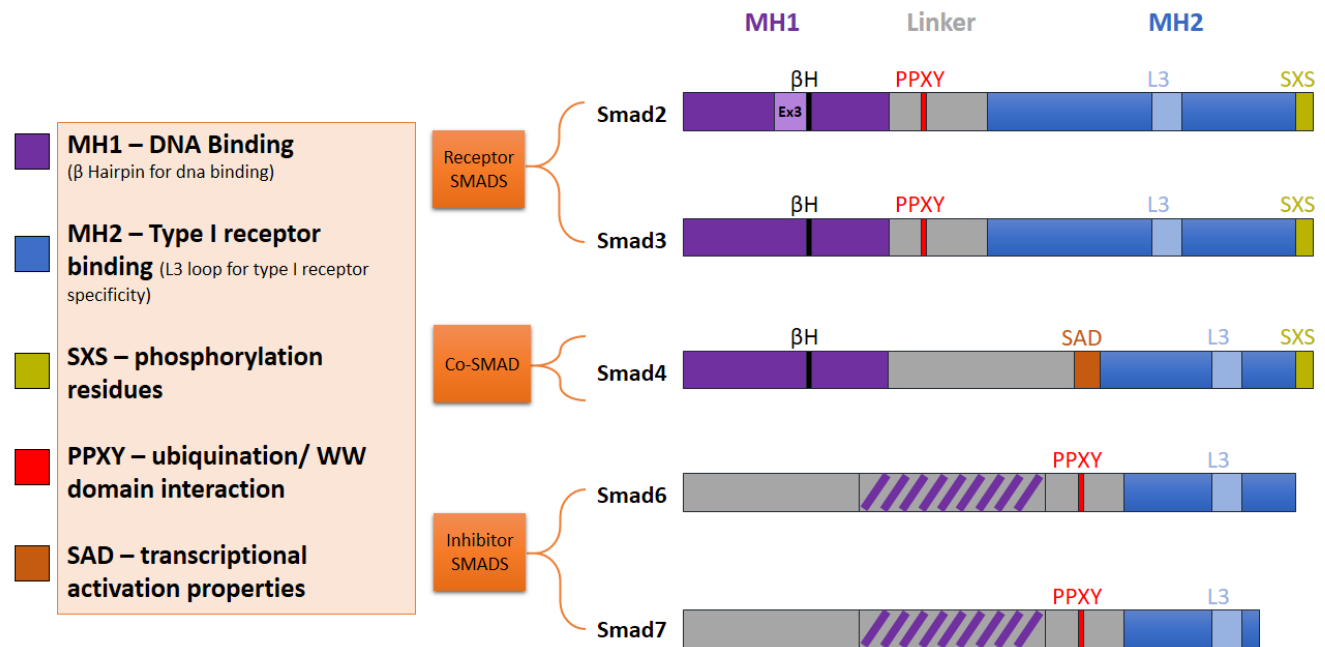


Figure 5: Schematic diagram of different domains in Smad proteins.

Upon activation of the receptor complex, R-Smads are recruited to the cytoplasmic tail of the type I receptor, at which both serines in the SXS motif in the C-terminus end of the R-Smads are phosphorylated. This causes a conformational change that dissociates the R-Smads from the receptor complex and the phosphorylated R-Smads bind to the Co-Smad, Smad4, forming what is termed the RC-Smad complex. TAZ (Transcriptional co-activator with PDZ-binding motif) is also recruited to the RC-Smad complex and is suggested to be required for the translocation of the RC-Smad complex into the nucleus (Varelas et al., 2008) (**Figure 6**).

Once in the nucleus, the RC-Smad complex is able to bind to its consensus DNA sequence (GTCTAGAC) with, Smad3 in particular, binding to the Smad Binding Element (SBE) sequence (5' GTCT 3') (Shi et al., 1998). Interestingly, the interaction between the RC-Complex

and the SBE is weak, which is why the target promoters usually have the SBE adjacent to binding sites of other transcription factors that associate with the RC-Smad complex. The RC-Complex can interact with a wide variety of DNA binding transcription factors, transcriptional co-activators and co-repressors, which is why the context in which the TGF β signalling pathway is activated dictates the cellular response (Shi and Massagué, 2003, Heldin et al., 1997, Massagué, 1996).

For example, the transcriptional activation properties of the R-Smads relies on the recruitment of CBP (CREB binding protein/p300) (Feng et al., 1998, Guannan et al., 2005). Along with CBP, the RC-Smad complex containing Smad2/3 recruit P/CAF (P300/CBP associated factor) and GCN5 while only GCN5 is recruited to the RC-Smad complex containing Smad1, 5, 8 (Itoh et al., 2001, Kahata et al., 2004). Another example of the diversity seen in interacting partners of the RC-Smad complex is the recruitment of BRG1 that is required for the upregulation of TGF β response genes in epithelial cells. The BRG1-RC-Smad complex is able to recruit the SWI/SNF complex to the target promoter and this recruitment is required to initiate target gene expression (Xi et al., 2008).

Inhibitory I-Smads

An interesting feature of this signalling pathway is the existence of inhibitory Smads (I-Smads; Smad6 and Smad7) that repress the canonical TGF β pathway by physically binding to the activated cytoplasmic tail of the Type I receptor, while being prone to phosphorylation due to a lack of the SXS motif seen in R-Smads (Hayashi et al., 1997, Goto et al., 2007) (**Figure 5**).

Since I-Smads lack the phosphorylation motif, they are unable to undergo the same conformational change that R-Smads require to dissociate from the receptor complex. As a

result, I-Smads inhibit the formation of the RC-Smad complex and the subsequent downstream effects by inhibiting the TGF β receptor activity (Shi and Massagué, 2003).

Smad6 in TGF β signaling

Smad6 is the inhibitory Smad for the BMP Signalling pathway, binding to the type I BMP receptors and blocking phosphorylation of Smad1, and 2. However, an alternative inhibitory mechanism was suggested by another group where Smad6 doesn't have an effect on the phosphorylation of Smad1 and binds to Smad4 instead, preventing the formation of the RC complex. Additional mechanisms aside from inhibiting the BMP receptor complex have been proposed for Smad6 with studies indicating an interaction with the glucocorticoid receptor (GR) and subsequent recruitment of HDAC3, resulting in suppression of any GR facilitated transcriptional activity. Furthermore, Smad6 was shown to bind to DNA and recruit Class I HDACs, inhibiting expression of BMP stimulated genes (Goto et al., 2007).

Smad7 in TGF β signaling

Smad7, another I-Smad, which structurally resembles Smad6, also has a highly conserved MH2 domain and much like Smad6, has a much less conserved MH1 domain (Hayashi et al., 1997). Although Smad7 hinders the type I BMP receptor's ability to phosphorylate R-Smads, its canonical role is to inhibit the TGF β /Nodal Signalling by binding to the TGF β type I receptor, preventing Smad2, and Smad3 phosphorylation. Smad7 interacts with the TGF β type I receptor and with Serine/threonine kinase receptor associated protein (STRAP), interfering with the physical access of Smad2/3 to the activated TGF β receptor complex (Datta and Moses, 2000, Datta et al., 1998). The C-terminus of the Smad7 protein is essential for the interaction with the type I TGF β receptor (Mochizuki et al., 2004). Pyruvate dehydrogenase Kinase 1 (PDK1) also

binds to Smad 2, 3, 4 and 7 and enhances PDK1 kinase activity but reduces the transcriptional properties of the R-Smads which suggests that Smad7 and PDK1 might be working together to inhibit TGF β signalling pathway (Seong et al., 2005, Seong et al., 2007).

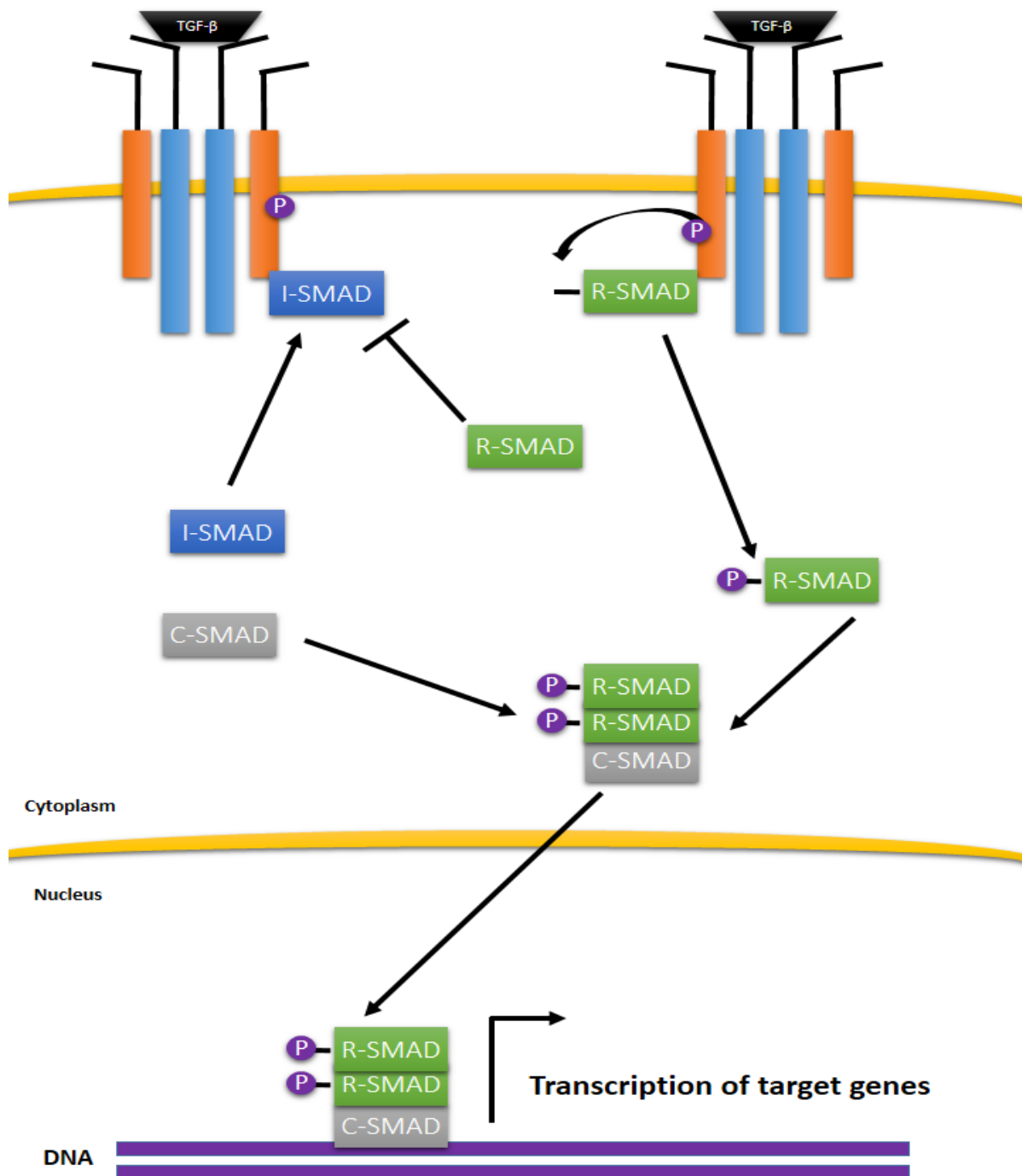


Figure 6: Schematic Diagram of Canonical TGF β signalling through Smad proteins.

Smad independent, ‘noncanonical’ TGF β Signalling pathways

Besides the canonical TGF β signalling pathway that involves activation of R-Smads, TGF β signalling has also been implicated in the regulation of many other signalling pathways including the MAPK and and activate kinase 1 (TAK1) signalling pathway (Shibuya et al., 1996, Yamaguchi et al., 1995). TAK1 was shown to bind to the unstimulated TGF β type I receptor and upon ligand stimulation, TAK1 dissociates from the receptor and autophosphorylates to become activated. Since TAK1 is an MKKK, it can activate MKK (Ishitani et al., 1999, Ninomiya-Tsuji et al., 1999). TGF β was also shown to activate all three branches of MAPK pathways, p38 MAPK, JNK, and ERK (Hocavar et al., 2005). TGF β induction of ECM production and fibrosis was shown to be through TGF β -induced activation of the MKK3-p38 MAPK and MKK4-JNK signalling pathways and thus subsequent type I collagen and fibronectin expression respectively (Zhang et al., 2000).

Smad7 regulation

The expression of the Smad7 gene is enhanced upon TGF β stimulation, creating a negative feedback loop for the canonical TGF β pathway (Nakao et al., 1997). Ectopic Smad3 is able to upregulate Smad7 expression by binding to the SBE in the regulatory region of the *smad7* gene (Denissova et al., 2000). The *smad7* gene was also shown to be regulated by AP-1 and Sp1 in a Smad2/3 dependent way (Brodin et al., 2000). Upon TGF β stimulation, the transcription factor IGHM enhancer-3 (TFE3) was shown to synergistically upregulate the Smad7 gene with ectopic Smad3 through the binding of Smad3 to the SBE, and the recruitment of TFE3 to an E-box in close proximity to the Smad3 bound SBE (Hua et al., 2000, Stopa et al., 2000).

Smurf2, Tiul, and NEDD4-2 are all E3 ubiquitin ligases that bind to Smad7 and enhance its inhibitory effect on TGF β signalling by targeting the Smad7 bound receptor complex for

degradation (Chong et al., 2006, Murakami et al., 2003, Kavsak et al., 2000, Kuratomi et al., 2005, Seo et al., 2004). AIP4 and Arkadia which are also E3 ubiquitin ligases, bind to Smad7, but can only target the Smad7 protein for degradation (Lallemand et al., 2005, Niederländer et al., 2001). AIP4 and Arkadia are therefore enhancers of the TGF β signalling pathway because of their ability to target Smad7 for ubiquitination.

Smurf1 E3 ligase can also bind to Smad7, even when Smad7 is bound to the TGF β type I receptor. This helps bring Smurf1 to close proximity of the TGF β type I receptor and target the TGF β receptor-Smad7 complex for degradation (Tajima et al., 2003). However, Smad7 itself can also be ubiquitinated by Smurf1, even if not bound to the TGF β receptor (Suzuki et al., 2002). Furthermore, Smad7 can also act as a bridging protein for Smurf1 and Smad4, allowing Smurf1 to also ubiquitinate Smad4 and target it for degradation. Smurf1 can therefore be viewed as both an activator, and as a repressor of the TGF β pathway, depending on the cellular context (Morén et al., 2005).

Another way that Smad7 is able to inhibit TGF β receptor complex activity is by binding to the type I receptor, and recruiting the Growth arrest and DNA damage-34 (GADD34) protein. GADD34 is part of the Protein phosphatase 1 protein, which was shown to dephosphorylate, and hence deactivate the TGF β type I receptor (Shi et al., 2004).

The Smad7 protein can also be regulated via PTM. P300 was shown to acetylate lysine residues 64 and 70 and prevent Smurf1 mediated ubiquitination of the same residues, allowing stabilization of the Smad7 protein (Grönroos et al., 2002).

Smad7 sub-cellular localization

Interestingly, Smad7 was shown to be predominantly localized in the nucleus of fibroblast-like COS1 cells and upon TGF- β 1 stimulation, Smad7 accumulated in the cytoplasm.

The study found that the MH2 domain for Smad7 was required for nuclear localization (Itoh et al., 1998, Hanyu et al., 2001). However, another group showed that TGF β stimulation had no effect on subcellular localization of Smad7 in epithelial-like Mv1Lu cells, but subcellular localization would depend on the material the cells were cultured in. If the cells were cultured on fibronectin coated glass plates or plastic, then Smad7 would predominantly localize in the cytoplasm. However, if the cells were cultured on a glass bottom plate, Smad7 would localize predominantly in the nucleus (Zhu et al., 1999).

As mentioned before, Smurf1 is able to ubiquitinate Smad7 regardless if its bound to the receptor or not. Ebisawa *et al.* found that smurf1 mediated ubiquitination of Smad7 can induce its translocation into the cytoplasm via a nuclear export receptor termed exportin-1 (CRM-1) (Ebisawa et al., 2001). CRM-1 mediated export of Smad7-Smurf1 complex requires the NES of Smurf1. Much like Smurf1, Tiul1 was also shown to co-translocate with Smad7 from the nucleus into the cytoplasm (Komuro et al., 2004). PDK1 was also shown to interact with Smad7 and inhibit its nuclear export (Seong et al., 2007).

Nuclear function of Smad7

A number of roles for Smad7 in the nucleus have been reported. Smad7 was shown to associate with HDAC-1 in NIH 3T3 fibroblasts, and was able to suppress E2F1 transcriptional activity through HDAC-1, suggesting Smad7 as a corepressor of E2F1 (Emori et al., 2012). Interestingly, ectopic expression of Smad7 was shown to induce cell cycle arrest in a variety of mesenchymal cells through down-regulation of G1 cyclins (Emori et al., 2012). Smad7 was shown to bind to SBE in the promoter of the *plasminogen activator inhibitor-1 (pail)* gene via its MH2 domain and prevent R-Smad dependent transcription of *pail* (Zhang et al., 2007). Our group previously showed that Smad7 physically binds to and enhances MyoD's transcriptional

properties, promoting skeletal muscle differentiation independent of its inhibitory role on the TGF β signalling pathway (Miyake et al., 2010) (**Figure 7**).

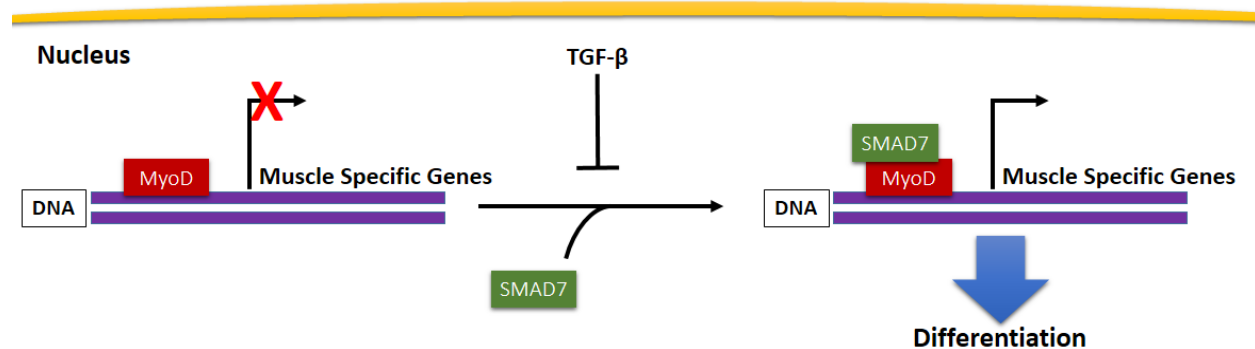


Figure 7: Schematic diagram of Smad7 nuclear function in skeletal muscle differentiation.

Pathophysiological roles of Smad7

Scleroderma, also known as systemic sclerosis, is a chronic systemic autoimmune disease leading to excessive fibrosis in the affected internal organs. Sample tissues have shown hyperactive TGF β , Smad2 and Smad3 activity, combined with reduced levels of Smad7. Exogenous expression of Smad7 attenuates the chronic activation of the TGF β signalling (Dong et al., 2002). In Tubulointerstitial fibrosis, Smurf1 and Smurf2 are upregulated, and as mentioned before, one of their target substrates is Smad7. The upregulation of Smurf1 and -2 reduces Smad7 levels while enhancing presence of phosphorylated Smad2 and -3 in the nucleus (Fukasawa et al., 2004). In contrast, Smad7 is hyperactive in inflammatory bowel disease (IBD), causing upregulation of pro-inflammatory cytokines which would normally be downregulated via TGF β signalling (Monteleone et al., 2001). One of the downstream targets of the TGF β signalling pathway is NF-KB, a key transcriptional factor that upregulates pro-inflammatory cytokines. TGF β stimulation induces reduction of NF-K β levels. However, in IBD, TGF β signalling can no longer reduce NF-K β levels due to high levels of Smad7 (Monteleone et al., 2004).

Laminin $\alpha 2$ -deficient congenital muscular dystrophy type 1A (MDC1A), an autosomal muscle disease that leads to muscle waste and shortened lifespan. Treatment with Losartan, an angiotensin II type I receptor blocker, restored some skeletal remodeling while reducing fibrosis (Mehuron et al., 2014). The mechanism suggested for Losartan was by inhibition of the TGF β signalling pathway that resulted in reduced levels of phosphorylated Smad2, -3, while also showing decreased TGF- β 1 ligand levels. Moreover, Losartan treatment led to higher levels of the Smad7 protein (Elbaz et al., 2012).

Role of TGF- β signaling in myogenesis

In vivo experiments suggest that TGF β prevents premature differentiation of migrating progenitor myoblasts, allowing sufficient proliferation of myoblasts before differentiation of limb muscle in the developing embryo. Large amounts of BMP2, one of the TGF β ligands, were found in the limb bud along with the ectoderm. Moreover, BMP2 was shown to inhibit the differentiation of secondary myoblasts but not the differentiation of embryonic myoblasts (Yanagisawa et al., 2001). Furthermore, cell culture experiments revealed that TGF β is a potent inhibitor of myoblast differentiation (Olson et al., 1986). TGF β stimulation led to reduced cell fusion and reduced expression of myogenic markers, troponin T and α -actin while fibronectin and collagen type I was upregulated (Massagué et al., 1986). If however, TGF β stimulation was removed, the cells would immediately express myogenic related genes and undergo normal differentiation (Massagué et al., 1986, Olson et al., 1986). It is important to note that after the onset of differentiation stimulation, the cells would become resistant to the antagonistic effect of TGF β on myoblast differentiation (Florini et al., 1991). Interestingly, the effect TGF β has on myogenesis was shown to be dependent on cell culture density. The higher the density, the larger the inhibitory effect of TGF β on differentiation, and the myoblasts would undergo differentiation

if the cells were cultured at low density or co-cultured with non-mesenchymal cells, such as C5 epithelial cells, even with presence of TGF β (Zentella and Massague, 1992, De Angelis et al., 1998). TGF β was shown to inhibit the transcriptional activity of Myogenin and another group showed that upon TGF β stimulation, MEF2C was exported out of the nucleus while the MRFs localized in the nucleus (Brennan et al., 1991, De Angelis et al., 1998).

Furthermore, Smad3 alone was shown to bind to MyoD, on the HLH domain that is required for association with its E-protein co activators such as E12 and E47 (Liu et al., 2001). However, since Smad7 is not able to reverse the inhibitory effect of TGF β on myogenesis, the complete intracellular mechanism by which TGF β inhibits myogenesis has yet to be elucidated, even after almost four decades since the first cell culture effects were described.

It has been suggested that TGF β represses myogenesis through the canonical TGF β /Smad3 pathway. However, there are three reasons why the canonical TGF β /Smad3 pathway cannot be the only intracellular mechanism for this inhibitory effect. Firstly, Smad3 inhibition by introduction of a Smad3 specific inhibitor (SIS3) was not able to reverse the effect of TGF β on myogenesis. Secondly, even though Smad7 is a potent inhibitor of Smad3 activity, introduction of Smad7 does not reverse the inhibitory effect of TGF β on myogenesis (Kollias et al., 2006). Lastly, introduction of a MEK inhibitor reverses the inhibitory effect of TGF β on myogenesis, suggesting a non-canonical pathway independent of the R-Smads (Miyake et al., 2009).

Role of Smad7 in myogenesis

Our group showed that Smad7 is required for the normal course of differentiation in C2C12 myoblasts. siRNA mediated depletion of endogenous Smad7 led to attenuated levels of

MyoD, Mef2A, MyHC, and Myogenin levels, all of which increase in the normal course of differentiation. Smad2, -3, and -4 protein levels and Smad2, -3 phosphorylation levels were not affected even though exogenous Smad7 is able to repress Smad2, and -3 transcriptional activity. Furthermore, the ectopic expression of Smad7 was able to attenuate the inhibitory effect of myostatin (a member of the TGF β ligand superfamily), but not TGF β on myogenesis which means that TGF β inhibits myogenesis through a Smad2/3 independent mechanism (Kollias et al., 2006).

In addition, exogenous Smad7 is not only able to reverse the inhibitory effect of Myostatin on myogenesis, it also enhanced expression of myogenic markers and myotube formation (Kollias et al., 2006). Furthermore, our group provided further evidence that exogenous nuclear Smad7 is sufficient for promoting myogenesis, independent of its role with TGF β at the receptor level (Miyake et al., 2010).

Cohen *et al.* investigated the loss of function of Smad7 *in vivo* by generating a Smad7 knockout mice. Smad7(-/-) mice showed hypotrophic, underdeveloped muscle fibres, resulting in reduced muscle mass and force generation compared to wild-type mice. Moreover, muscle regeneration was delayed in Smad7(-/-) mice due to reduced satellite cell activation (Cohen et al., 2015).

Smad7 was shown to directly bind to, and protect MyoD from the repressive effects of MEK, suggesting a nuclear coactivator role for Smad7, which is independent of its antagonistic role on TGF β signalling (Kollias et al., 2006). It is important to further explore Smad7's function in the nucleus for intracellular mechanisms that result in the effect of Smad7 on myogenesis.

Epigenetic changes during skeletal muscle differentiation

Numerous covalent modifications have been directly regulate the transcriptional state of genes, such as the marks H3K4me3 and H3K9Ac, which have been associated with decondensation of DNA, allowing physical access transcription factors and transcription of nearby genes, and H3K27me3 which is associated with transcriptional repression of genes in proximity of the histone mark (Zhang et al., 2015, Blais, 2015).

ChIP-seq experiments comparing C2C12 myoblasts and myotubes revealed many histone mark changes at muscle specific transcriptional enhancers, the majority of which were MyoD dependent, because these histone mark changes were reduced in MyoD depleted conditions in myoblasts (Asp et al., 2011, Vethantham et al., 2012, Blum et al., 2012, Cheng et al., 2014). MyoD was shown to play an active role in forming the landscape of histone marks on enhancers through Setd7. Setd7 is an H3-lysine 4 specific methyltransferase and an important enzyme required for normal myogenesis (Tao et al., 2011).

H3K4me1 was shown to be a transcriptionally repressive marker in the context of myogenesis as the reduction in this mark led to an upregulation of muscle specific genes (Cheng et al., 2014). At the onset of differentiation, in the normal course of myogenesis, the H3-lysine-4 specific demethylase, Lsd1 has been shown to remove the repressive H3K4me1 mark at muscle specific enhancer regions, paving the way for MLL1, a H3 methyltransferase, to trimethylate H3K4. The trimethylation of H3K4 cannot be reversed by Lsd1, resulting in the transcriptional activation of the genes examined. Interestingly, H3K4me3 is recognized by the Inhibitor of Growth 1 (ING1), which recruits an Lsd1/Sin3a complex that can antagonize any further H3K4me1 marks in proximity (Cheng et al., 2014).

In contrast to H3K4me3, H3K27me3 represses transcription of nearby genes. The catalytic subunit of Polycomb Repressive Complex 2 (PRC2), Ezh2, was shown to antagonize muscle gene induction, and as a result, inhibit myogenic differentiation (Carette et al., 2004). The transcription factor Yy1 was shown to recruit Ezh2 to muscle specific genes to induce H3K27 trimethylation before differentiation. However, Ezh2 and Yy1 are removed from the muscle specific genes at the onset of differentiation, resulting in lower levels of H3K27me3 and transcriptional activation of the genes (Lu et al., 2013).

Furthermore, the enzyme Lysine Demethylase 6A (KDM6A) was shown to be recruited by the histone chaperone Spt6 and demethylate H3K27 (Wang et al., 2013). Spt6 is also reported to assist passage of RNA pol II through destabilization of nucleosomes. Knockdown of Spt6 results in increased levels of H3K27me3, reduced expression of muscle specific genes, and inhibition of myogenesis (Seenundun et al., 2010).

Statement of Purpose

Our group previously showed that introduction of exogenous Smad7 in C2C12 cells induced early myogenesis by amplifying expression of MRFs. Smad7 was shown to cooperate with -and promote- MyoD driven myogenesis (Kollias et al., 2006). A follow up study revealed exogenous nuclear Smad7 had the capacity to induce an equivalent effect on myogenesis, ruling out Smad7's well characterized cytoplasmic role as the main underlying mechanism for the observed effects (Miyake et al., 2010).

A recent screen for Smad7 interacting proteins indicated an interaction between Smad7 and SET/TAF-I β , a nuclear localized proto-oncogene which plays diverse functions in chromatin remodeling, cell cycle, autophagy, and apoptosis. SET is a major subunit of the INHAT (Inhibitor of Histone Acetylation) complex capable of binding to – and masking – histones from acetylation, particularly H3 and H4 tails, resulting transcriptional inhibition of nearby genes.

We speculated that SET may play a major role in the epigenetic changes required for myogenesis by functioning as a transcriptional inhibitor of muscle specific genes. We hypothesized that Smad7 antagonizes this repressive effect of SET on muscle specific genes, resulting in early myogenesis.

Exploring the role of SET and Smad7 interaction in skeletal myogenesis is pivotal to our understanding of skeletal muscle ontogeny and physiology.

Introduction

Skeletal muscle is an essential tissue that allows locomotion and supports proper posture for vertebrates. Skeletal muscle differentiation (myogenesis) is the process of converting mono-nucleated myoblasts into multi-nucleated myotubes that form the basis of musculature. Myogenesis requires a highly coordinated program of gene expression that involves MRF and MEF2 transcription factors as major orchestrators inducing muscle specific gene expression. The TGF β signaling pathway plays an essential role in myogenesis, inhibiting premature differentiation of myoblasts to ensure sufficient number of myoblasts are present before fusion and differentiation into myotubes. A major inhibitor of the canonical TGF β signaling pathway is Smad7 which binds to the activated TGF β receptor complex and inhibits downstream signaling. Interestingly, exogenous wildtype Smad7 does not reverse the inhibitory effect of TGF β on myogenesis indicating a different, non-canonical downstream signaling pathway as the underlying mechanism.

Our group recently showed that exogenous nuclear Smad7 is capable of inducing premature myoblast differentiation, indicating a noncanonical role for Smad7 in myogenesis. Smad7 was shown to form a mutually reinforcing positive feedback loop with MyoD, with Smad7 protecting MyoD transcription activity from the repressive effects of Mek, and MyoD promoting Smad7 expression. However, further elucidation of the nuclear role of Smad7 is needed, especially how Smad7 activity is regulated in the nucleus. A recent screen for Smad7 interacting partners indicated an interaction between Smad7 and SET/TAF-I β , a nuclear proto-oncogene involved in diverse functions such as chromatin remodeling, histone masking, and cell cycle (unpublished data).

Here we show that SET is capable of inducing epigenetic changes leading to transcriptional repression of myogenic related genes such as *mck* and *myogenin*, and ‘anti’-myogenesis genes such as *cyclin D1*. We show that Smad7 is able to bind to, and antagonize SET’s repressive effect on myogenic related genes, but not *cyclin D1*. SET mediated repression of the cell cycle coupled with Smad7’s antagonism of SET at myogenic genes results in a pronounced enhancement of myogenesis compared to Smad7 enhancement alone.

Materials and Methods

Plasmids

WT-myc-Smad7 and myc-Smad7-NLS expression vectors were described previously (Miyake et al., 2010, Kollias et al., 2006). Luciferase reporter constructs, pMCK-luc (Donoviel et al., 1996) and pMyoG-luc (Miyake et al., 2010) were described elsewhere. Flag-SET expression vector (#24998) was purchased from Addgene.

Antibodies

Mouse IgG (sc-2025), dsRed (sc-33354), MCK (sc-365046), Actin (sc-1616) antibodies were purchased from Santa Cruz Biotechnology. FLAG(A-9594) antibody was purchased from Sigma. H3K9ac(ab4441), H3(ab1791), H3K4me3(ab8580), H3K27me3(ab6002) antibodies were purchased from Abcam. Myogenin (F5D), Myc (9E10), and MyHC (MF20) antibodies were from Dako Cytomation. Rabbit IgG (12-370) was from Millipore.

Cell Culture

C2C12 and HEK293T cells were purchased from American Type Culture Collection. The cells were all cultured in 10% Fetal bovine serum (FBS) (HyClone) in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Hyclone) with 1% penicillin-streptomycin (Invitrogen) at 5% CO₂ and at 37°C. The differentiation conditions were in 2% horse serum (Atlanta Biologicals) in high glucose DMEM supplemented with 1% penicillin-streptomycin (Invitrogen).

Western blotting analysis

Conventional cell scrapers were used to collect cells in 1X cold PBS. NP-40 lysis buffer (0.1 % NP-40, 150 mM NaCl, 1mM EDTA, 50 mM Tris-HCl pH 8.0, 1mM sodium vanadate, 1mM

PMSF, supplemented with a protease inhibitor cocktail (Sigma, P-8340)) was used for biochemical lysis. Standard Bradford assay was used to determine protein concentrations (BioRad). Equal amounts of proteins were loaded on 10% SDS-PAGE gels and subsequent electrophoretic transfer was done on Immobilon-P membranes (Millipore). Blocking was done for 1hr in 5% milk in PBS, primary antibody was in 2% milk in PBS or Tris buffered saline (TBS)-T (10mM Tris-HCl pH8.0, 150mM NaCl, 0.1% Tween-20) incubated at 4°C overnight. HRP-conjugated secondary antibodies in 5% milk in PBS were incubated at room temperature for 1 hour (Santa Cruz Biotechnology, Cell Signaling Technology). Enhanced chemiluminescence reagent (Amersham) was used to detect immuno-reactive secondary antibodies still bound to the membrane. The membranes were then exposed to Biomax film (Kodak). All washes were done with 1X PBS.

Co-Immunoprecipitation assay

An equal amount of total cellular protein (250µg) was diluted with NP-40 lysis buffer to a final concentration of 1 µg/µl. Protein complexes were immunoprecipitated with the indicated antibody and 25µl of protein GPlus Sepharose beads (50% slurry) (Santa Cruz Biotechnology) by incubation at 4°C overnight on a rotating platform. The beads were washed three times with the following wash buffer (0.1% NP-40, 150mM NaCl, 1mM EDTA, and 50 mM Tris-HCl pH 8.0). Beads were boiled in SDS sample buffer, and protein complexes were resolved by SDS-PAGE and immuno-blotted as described above.

Reverse Transcription

Total RNA was extracted from cells using the RNeasy Plus kit (Qiagen) and Qias shredder (Qiagen). RNA was converted to cDNA using Superscript III (Invitrogen) according to the manufacturer's instructions.

Quantitative PCR

SybrGreen (BioRad) was combined with 2.5 μ l cDNA and 500 nM primers in a final volume of 20 μ l. cDNA was diluted 1:10 prior to use. Each sample was prepared in triplicate and analyzed using Rotor-Gene Q (Qiagen). Parameters for qRT-PCR using BioRad: 30s 95°C, [5s 95°C, 30s 60°C] x 40 cycles. Parameters for ChIP-qPCR: 5min 95°C, [5s 95°C, 15s 60°C] x 40 cycles.

Fold change (qRT-PCR) was quantified using the $\Delta\Delta$ Ct method. Primers used in ChIP-qPCR and qRT-PCR are listed in **Supplementary Table S1** and **S2**.

Chomatin Immunoprecipitation

Methods were carried out as described previously, however a third IP Wash Buffer was added (IP Wash Buffer III; 20 mM Tris pH 8.1, 250 mM LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA). See **Supplementary Table S2** for primers used in ChIP.

MyHC Staining

C2C12 cells were washed with Phosphate buffered saline (PBS) (pH7.4) and fixed with 4°C paraformaldehyde (4%) for 10min at room temperature followed by membrane permeabilization by 90% methanol at 4°C for 10 min. The cells were blocked using 5% milk in PBS for 1 hour at 37 °C. Cells were incubated at room temperature with MF-20 (MyHC antibody) diluted in blocking buffer (2% milk PBS) for 1 hour. Cells were then washed three times with PBS and incubated for 60 min at room temperature with Horseradish peroxidase (HRP)-conjugated α -

mouse secondary antibody. The cells were washed three times with PBS and incubated in developer (0.6 mg/ml DAB, 0.1 % H₂O₂ in PBS) to detect MyHC by immunocytochemistry. Images were recorded with a microscope (Axiovert 35; Carl Zeiss MicroImaging) with 10X NA 0.25 Achrostat objective lenses with the Canon EOS D60 digital camera.

Quantification of Western Blots

Quantification of the western blots were carried out using ImageJ (1.8.0_77) analysis software following instructions described previously (Davarinejad, 2015).

Transfections

All transfections were carried out using conventional CaPO₄ transfection method (see extended MM for protocol).

Luciferase Assay

Cells were transfected via the CaPO₄ transfection protocol, followed by 24h recovery in 10% FBS DMEM. The cells were then washed with 1X cold PBS and were lysed using the luciferase lysis buffer (see Extended MM for buffer recipe). Aliquots were taken for each of the three replicates and luciferase activity was analysed using Lumat³ Berthold Technologies (LB 9508) per manufacturer's instructions. Luciferase substrate was purchased from Promega (E151A).

Results

Exogenous SET and Smad7 form a complex

To confirm the interaction between SET and Smad7, exogenous Flag-SET and myc-Smad7 were expressed in human embryonic kidney HEK293T cells. The cell lysate was subjected to immuno-precipitation analysis. **Figure 8B** shows presence of exogenous myc-Smad7 in the Flag-SET immuno-precipitate but not by normal IgG. **Figure 8C** indicates presence of Flag- in the myc-Smad7 immuno-precipitate but not the corresponding control IgG. The results here show an interaction between exogenously expressed SET and Smad7 in HEK293T cells.

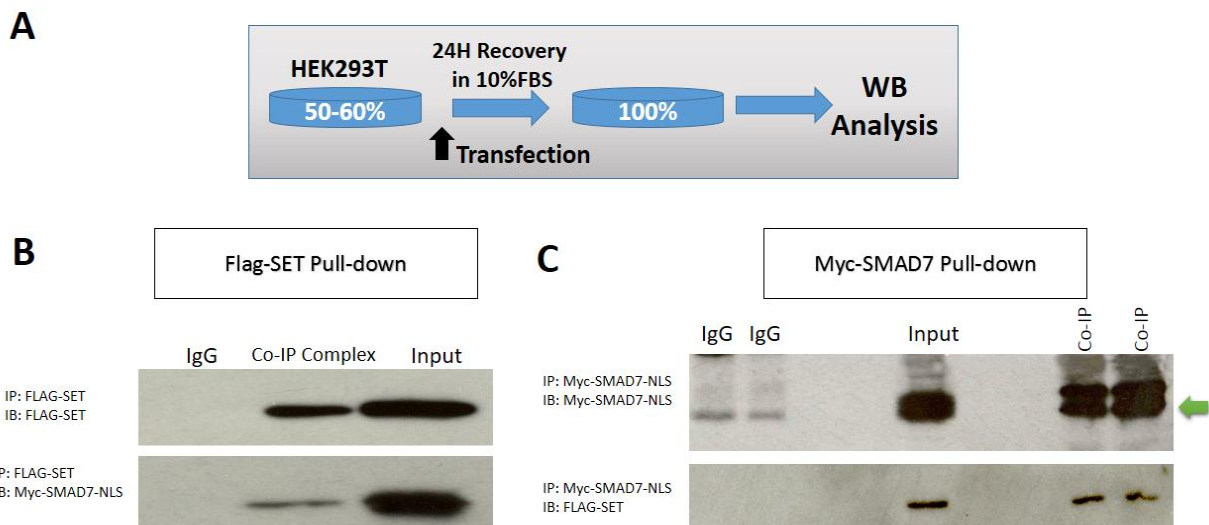


Figure 8: Exogenous SET and Smad7 interact in HEK293T cells. **A:** Cells were lysed biochemically (NP-40) and incubated with agarose beads conjugated with either Flag or Myc antibodies overnight at 4°C, followed by Western blot analysis. Mouse or Rabbit IgG was used as control for specificity of Smad7 and SET respectively. **A:** Diagram showing summary of experimental design. **B:** Blots showing successfully immunoprecipitated Flag-SET (top blot) and presence of myc-Smad7 in the immunoprecipitated complex (bottom blot) **C:** Blots showing successfully immunoprecipitated myc-Smad7 (top blot) and presence of Flag-SET in the immunoprecipitated complex (bottom blot).

Exogenous SET-Smad7 interaction induces premature differentiation

To investigate possible effects the SET-Smad7 interaction may have on myogenesis, exogenous SET and/or Smad7 were expressed in C2C12 cells, left to differentiate in low serum conditions for 72H, followed by immunostaining for MyHC to compare levels of myotube formation. **Figure 9C** shows a higher level of myotube formation when exogenous Smad7-NLS is expressed (bottom left) compared to pcDNA control, as reported previously (Kollias et al., 2006, Miyake et al., 2010). In contrast, exogenous SET expression induces cell-death indicated by floating round cells along with minimum myotube formation throughout the cultured dish (Top right). However, co-expression of SET and Smad7-NLS leads to an enhancement of myotube formation, with more robust myotubes formed compared to exogenous Smad7 alone. These results indicate that while SET alone inhibits myogenesis, when introduced with Smad7 together, leads to promotion of myogenesis and myotube formation, even more so than exogenous Smad7 alone. **Figure 9B** shows immunoblots of lysates from similarly transfected cells, indicating expression levels of myc-Smad7 and Flag-SET.

To examine whether the effects seen are due to deprivation of Smad7 from the cytoplasm, the experiment was repeated with wildtype-Smad7 with similar end results, shown in

Supplementary Figure 1.

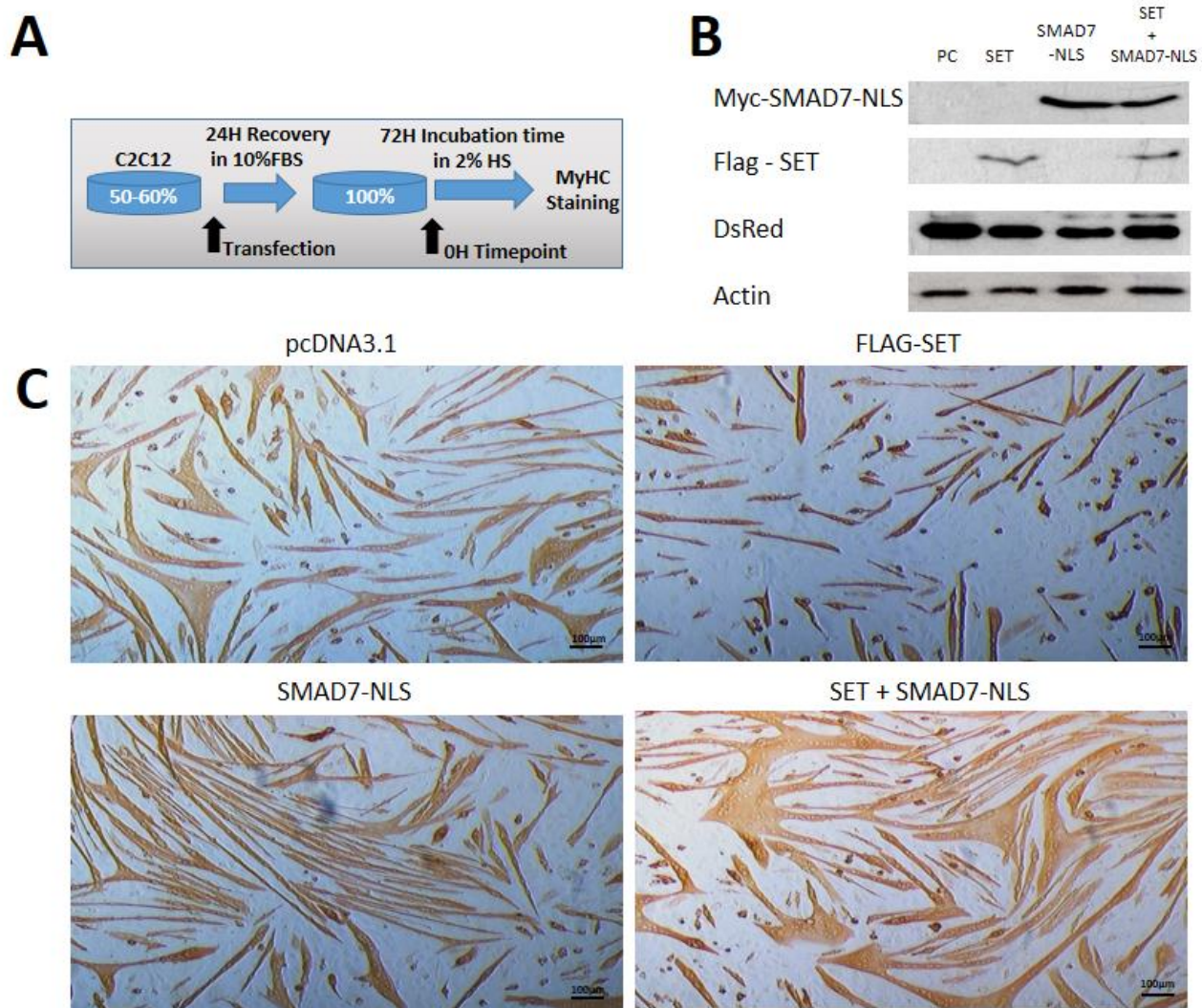


Figure 9: Exogenous SET and Smad7 promote myogenesis while ectopic expression of SET alone leads to cell death. C2C12 cells were transfected with Flag-Set and/or Smad7, left to differentiate for 72H in low serum conditions (2% HS) followed by 4% paraformaldehyde fixation and immunostaining MyHC. **A:** Diagram showing summary of experimental design. **B:** Western blot analysis using lysates of similarly transfected cells **C:** MyHC staining showing level of myotube formation.

Exogenous SET and Smad7 induce early expression of myogenic markers

To further investigate this phenotype induced by the SET and Smad7 interaction at a molecular level, exogenous SET and Smad7 were expressed in C2C12 cells and followed by Western blot analysis of myogenic markers in a differentiation timecourse. The resulting Immunoblots shown in **Figure 10** indicate a delayed MCK and MyoG expression with presence of exogenous Flag-SET. In-line with the previously reported data, expression of exogenous myc-Smad7 induced early MCK and MyoG expression when compared to control (Miyake et al., 2010, Kollias et al., 2006). However, co-expression of Flag-SET and myc-Smad7 led to an early amplification of MCK and MyoG expression, even more so than myc-Smad7 alone. Although exogenous Flag-SET alone repressed myogenic marker expression, exogenous co-expression of Smad7 and SET enhanced MCK and MyoG.

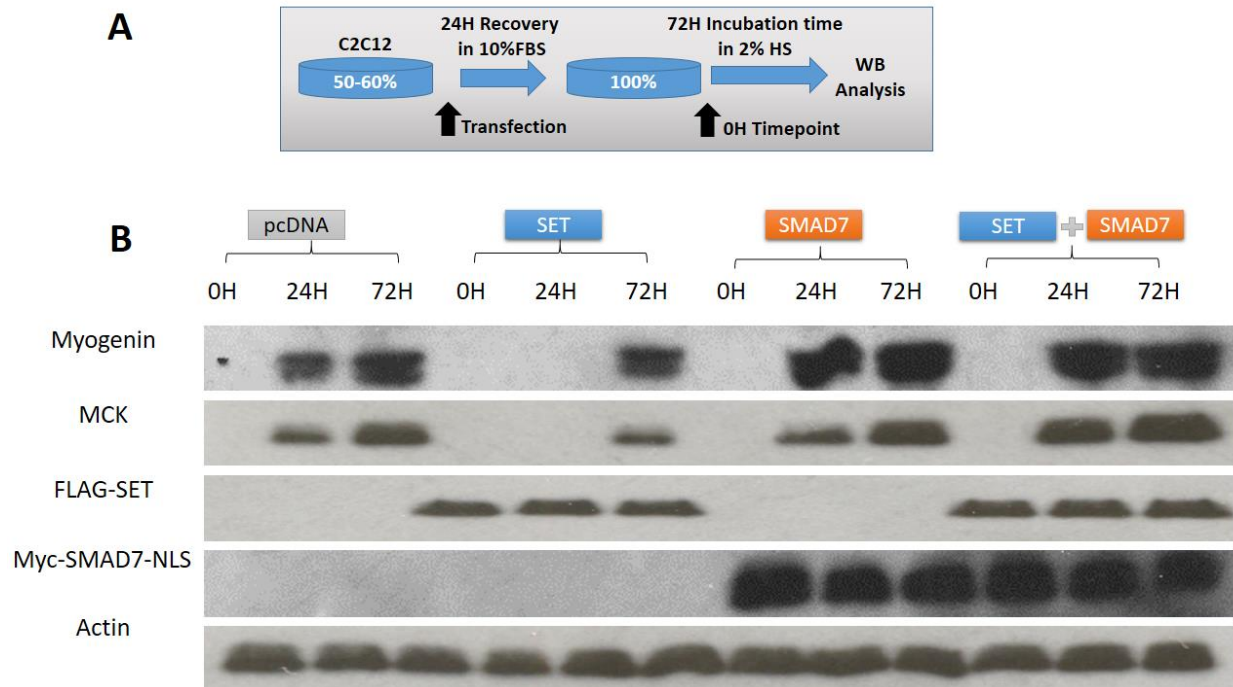


Figure 10: C2C12 differentiation timecourse with exogenous Smad7 and SET. C2C12 cells transfected with Smad7 and/or SET, left to recover in 10% FBS DMEM for 24H before biochemical lysis (NP-40) followed by Western Blot analysis. **A:** Diagram showing summary of experimental design. **B:** Western blots showing expression levels of the myogenic markers at various time points.

SET and Smad7 increase MCK and MyoG promoter activity

We decided to further investigate the promoter activity of the muscle marker genes in the presence of exogenous SET and/or Smad7 using luciferase reporter assays in C2C12 cells, shown in **Figure 11C**. Exogenous SET expression reduced *mck* promoter activity compared to that of control and while exogenous Smad7 enhanced the same promoter by ~3 fold, co-expression of exogenous SET and Smad7 together resulted in a near 5 fold increase in promoter activity within 24H after transfection. The *myog* promoter responded similarly to the *mck* promoter, with exception of when exogenous SET was expressed alone. The results here show an increase in the activity of both *mck* and *myog* promoter when exogenous SET and Smad7 are co-expressed.

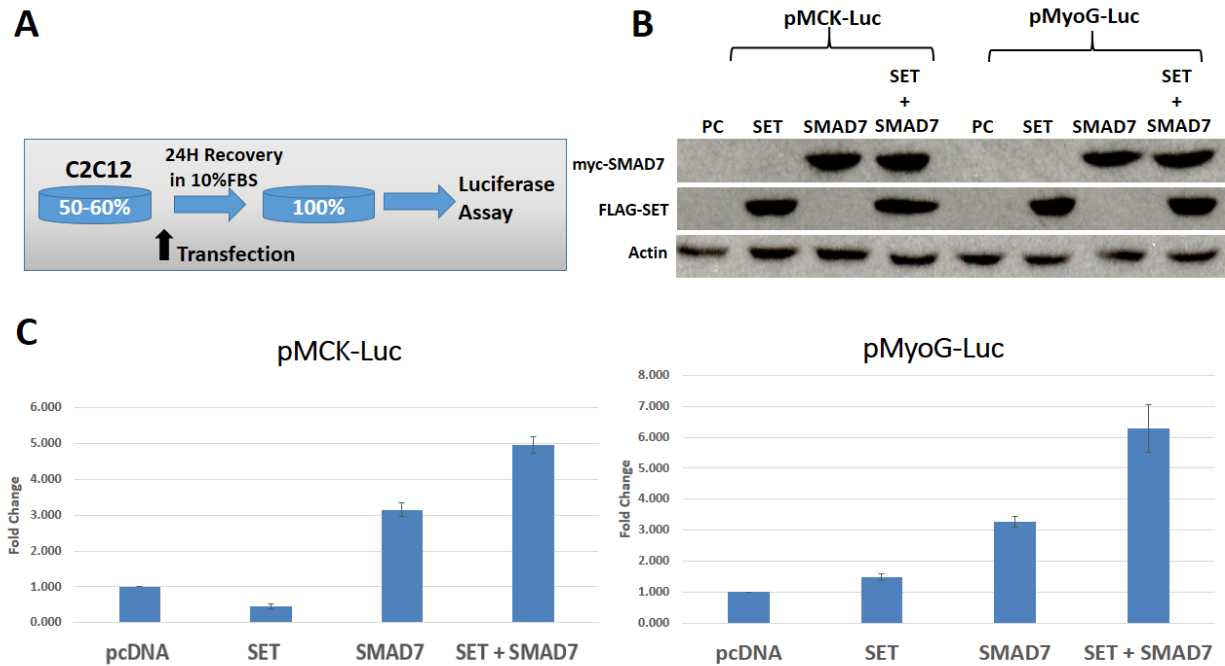


Figure 11: Luciferase assays of MyoG and MCK in the presence of exogenous SET and/or Smad7. SET and/or Smad7 were transfected into C2C12 cells along with either MCK (Left) or MyoG (Right) promoters fused to a luciferase reporter gene. **A:** Diagram showing summary of experimental design. **B:** Western blot showing expression levels of exogenous SET and Smad7. **C:** Results of the luciferase assay, with MCK-Luc (Left) or MyoG-Luc (right). Error bars represent SD values.

Endogenous mRNA levels of SET and Smad7 in a timecourse of C2C12 differentiation

To examine whether SET and Smad7 expression is changed during the C2C12 differentiation, we isolated total RNA from untransfected C2C12 cells in a timecourse, followed by RT-qPCR analysis shown in **Figure 12**. The results indicate peak endogenous Smad7 mRNA expression at the 24 hour timepoint, lowering after 72 hours in low serum. Interestingly, SET mRNA levels decrease throughout the differentiation process. The results here along with previous data indicate that reduction of SET expression is suitable for muscle differentiation. MCK and MyHC are expressed in later stages of differentiation at 72H, while Myogenin expression increases with time.

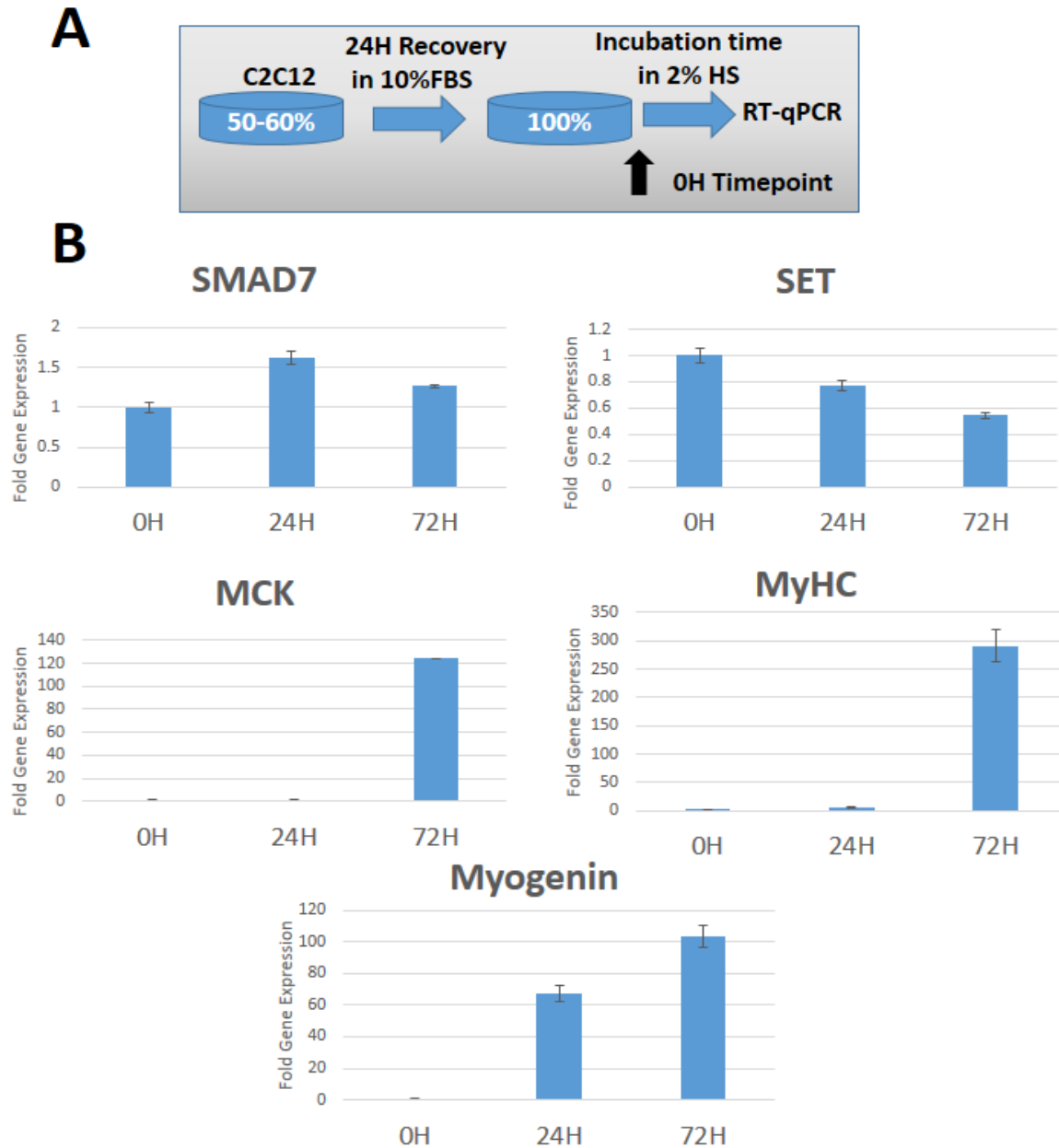


Figure 12: Endogenous mRNA levels of SET and Smad7 in a C2C12 differentiation timecourse. C2C12 cells were cultured in 10% FBS DMEM and switched to 2% HS DMEM for the stated duration once reaching confluency. Total RNA was then isolated using manufacturer's instructions (Qiagen) followed by standard RT-qPCR analysis. **A:** Diagram showing summary of experimental design. **B:** Results for qPCR reaction using primers against stated genes. Error Bars represent SD values.

Exogenous SET and Smad7 enhance myogenic marker expression at the promoter level

To confirm SET and Smad7 modulation of myogenic markers at mRNA level, exogenous SET and Smad7 were expressed in C2C12 cells, and mRNA levels of myogenic markers were quantified at various timepoints using RT-qPCR. The results shown in **Figure 13**, indicate an increase in mRNA levels of MCK, MyHC, and MyoG when exogenous Smad7 is present. In contrast, mRNA levels of all three markers decrease with expression of exogenous SET. However, exogenous expression of both SET and Smad7 resulted in a large increase in mRNA levels of the three myogenic markers analysed, showing a greater effect than that of exogenous Smad7 alone.

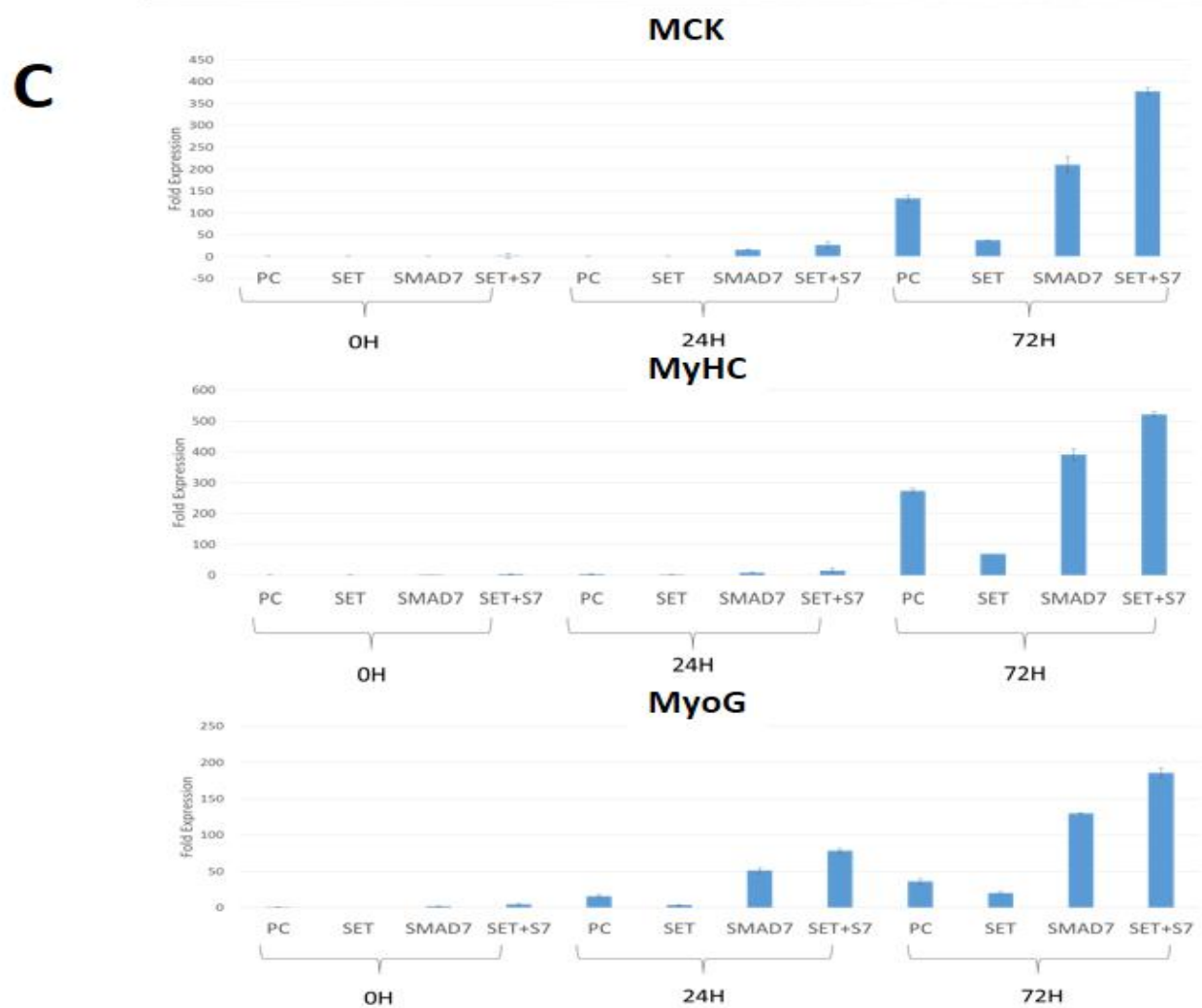
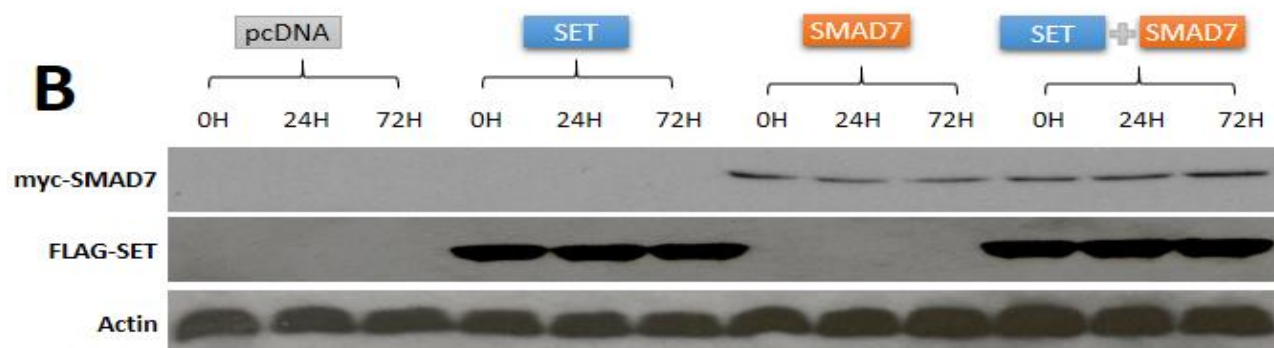
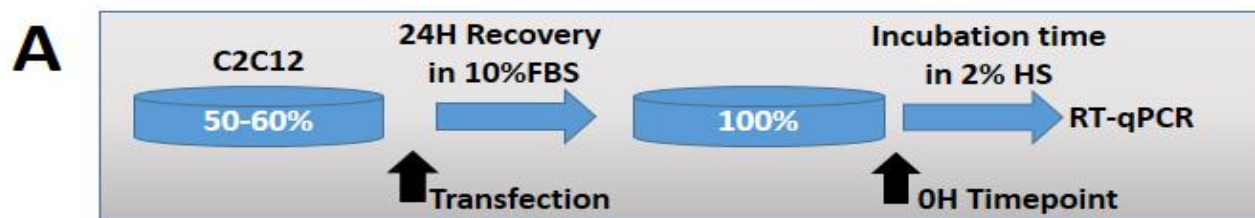


Figure 13: Myogenic marker mRNA levels in a C2C12 differentiation timecourse in the presence of exogenous SET and/or Smad7. C2C12 cells were transfected with SET and/or Smad7 and left to differentiate in 2% horse serum for the indicated hours before total RNA isolation and subsequent RT-qPCR analysis. **A:** Diagram showing summary of experimental design. **B:** Western blot analysis from lysates extracted from similarly transfected cells. **C:** RT-qPCR analysis shows mRNA levels of genes shown. Error Bars represent SD values.

Exogenous SET and Smad7 can alter total histone acetylation/methylation levels

SET is a major subunit of the INHAT complex capable of masking histone tails from acetylation. To investigate possible epigenetic modulations resulting from expression of exogenous SET and Smad7, we immunoblotted total cell lysates using antibodies specific to histone markers.

Figure 14 shows Exogenous SET expression induced large decreases in overall levels of both transcription activating marks (H3K4me3 and H3K9Ac), and a large increase in the transcription repressive mark H3K27me3 throughout the three timepoints when compared to the control pcDNA. In contrast, exogenous Smad7 induced an increase in levels of both activating marks H3K4me3 and H3K9Ac, and a small decrease in levels of repressive mark H3K27me3 when compared to control. Interestingly, when exogenous SET and Smad7 were co-expressed, there was a significant increase in total levels of activating marks H3K4me3 and H3K9Ac, and a significant decrease in levels of the repressive mark H3K27me3 when compared to pcDNA control, even more so than the effect of exogenous Smad7 alone.

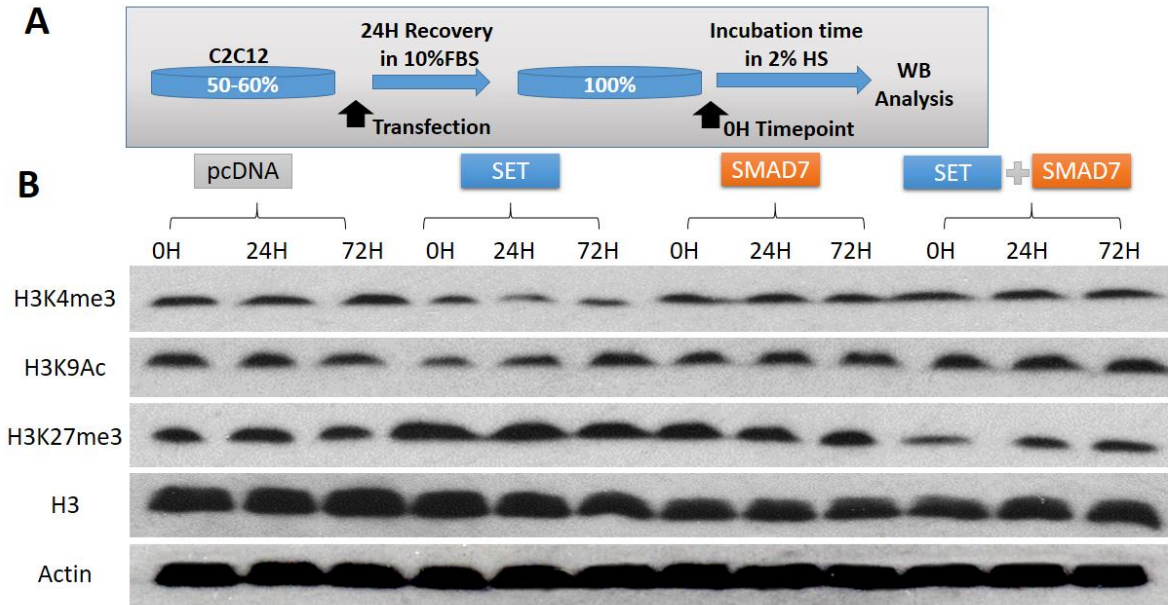


Figure 14: Modulation of total levels of histone marks H3K4me3, H3K9Ac, and H3K27me3 in the presence of exogenous SET and/or Smad7 in a C2C12 differentiation timecourse. C2C12 cells were transfected with either SET, Smad7, or SET+Smad7 and left to differentiate in 2% horse serum for the indicated hours. Western blots were carried out, loading total lysate and using antibodies against the indicated histone marks. H3K4me3 and K9Ac are known to generally activate gene transcription, while K27me3 represses gene transcription. **A:** Diagram showing summary of experimental design **B:** Immunoblots of the histone marks. Quantification of the western blots are shown in **Supplementary Figure 2**.

Smad7 inhibits the histone masking abilities of SET

To explore the interplay between SET, Smad7, and histone marks in the context of myogenesis, ChIP experiments were performed using histone mark specific antibodies with C2C12 cell lysates transfected with SET and/or Smad7 followed by a subsequent qPCR analysis of regions of interest. As shown in **Figure 15**, this experiment was designed with 4 different conditions: transfected pcDNA construct serving as control, transfected SET or Smad7 constructs alone, or transfected SET and Smad7 constructs together. Using antibodies specific to the histone marks, we analysed levels of the histone marks in the regions of interest shown in

Figure 15. H3K4me3 and H3K9Ac marks are known to be a transcription activating mark, while the H3K27me3 mark is generally a transcription repressive mark.

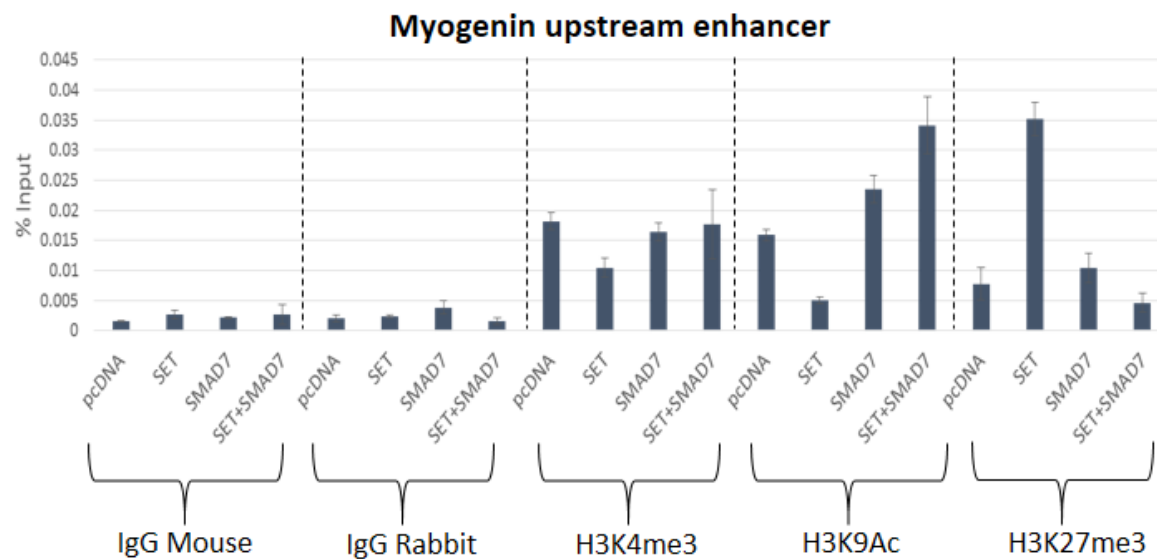
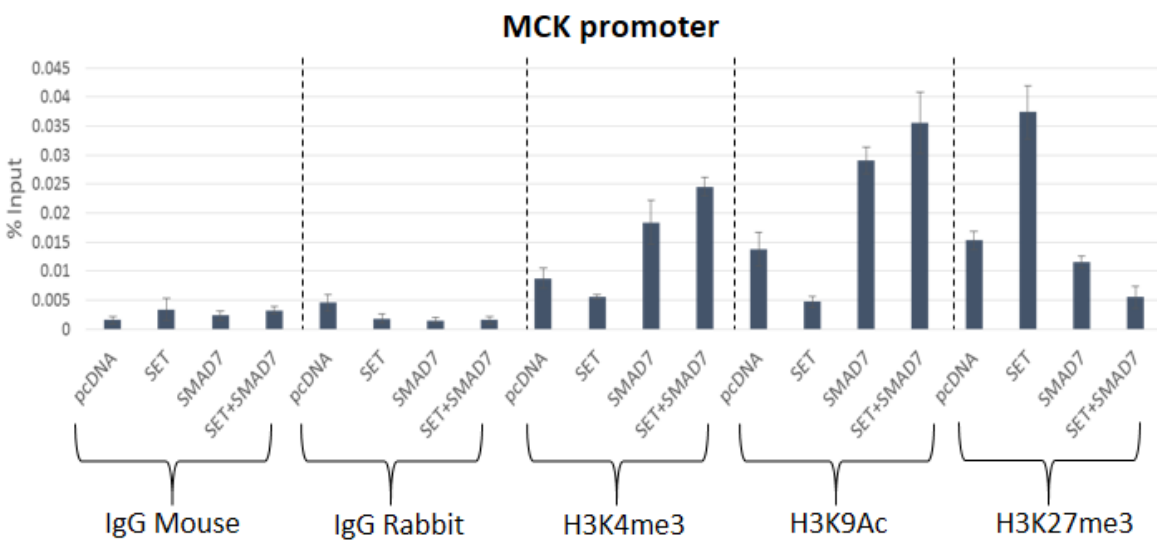
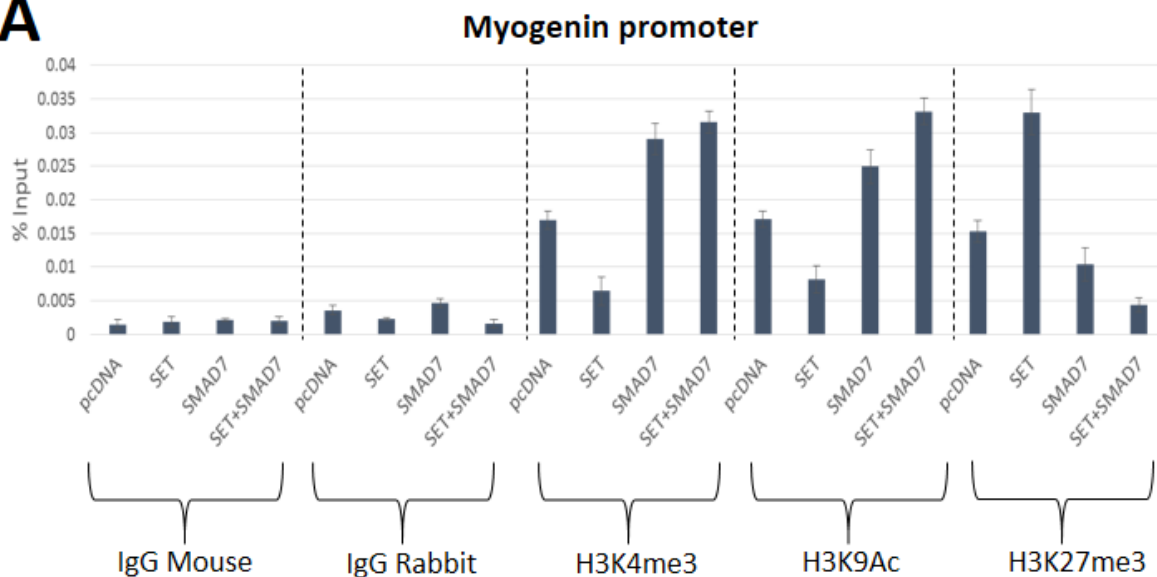
Exogenous expression of SET alone resulted in decreased levels of the H3K4me3 and H3K9Ac marks in proximity of the regulatory regions of all the genes examined with the exception of *gapdh*. This corresponds with previously reported literature of SET's histone masking properties. However, expression of exogenous SET also led to increased H3K27me3 levels in all the genes with the exception of *gapdh*. The constitutively active expression level of the *gapdh* gene requires maintenance of a constant 'open' DNA template state that does not include nucleosomes or histone proteins in proximity of its regulatory regions. This may explain why the epigenetic changes seen with introduction of exogenous SET does not apply to the *gapdh* gene. Overall, the results here suggest that SET functions as a transcriptional repressor for the *mck*, *myogenin*, and *cyclin D1* genes by inhibiting transcription activating marks such as the H3K4me3 and H3K9Ac and promoting H3K27me3 marks around the regulatory regions of those genes. However, due to the open DNA template state of *gapdh*, SET is not able to modulate GAPDH expression levels.

In contrast to the effects of SET, exogenous Smad7 induced lower levels of the repressive mark (H3K27me3), and higher levels of activating marks (H3K4me3 & H3K9Ac) on the regulatory regions of myogenic related genes examined. Smad7 is therefore a transcriptional activator to *mck* and *myogenin*, but has no effect on the epigenetic marks near the regulatory regions of either *cyclin D1* or *gapdh* genes.

The introduction of exogenous SET and Smad7 together resulted in higher levels of activating marks (H3K4me3/H3K9Ac) lower levels of the repressive mark (H3K27me3) in the regulatory regions of both *mck* and *myogenin* when compared to that of expression of exogenous

Smad7 alone. This result corresponds to the increased expression of the *mck* and *myogenin* genes shown in **Figure 13** and the increased MCK and MyoG protein levels shown in **Figure 10**.

Interestingly, SET's repressing role on the *cyclin D1* promoter is not effected when introduced together with exogenous Smad7. Unlike the Smad7 antagonizing effect of SET on the *mck* and *myogenin* genes, similar levels of both activating, and repressing histone marks are seen on the *cyclin D1* promoter regardless of the presence of exogenous Smad7. This suggests that Smad7 is able to target and inhibit SET's repressive effect on histones near regulatory regions of myogenic related genes but Smad7 does not modulate SET's repressive activity on the *cyclin D1* promoter.

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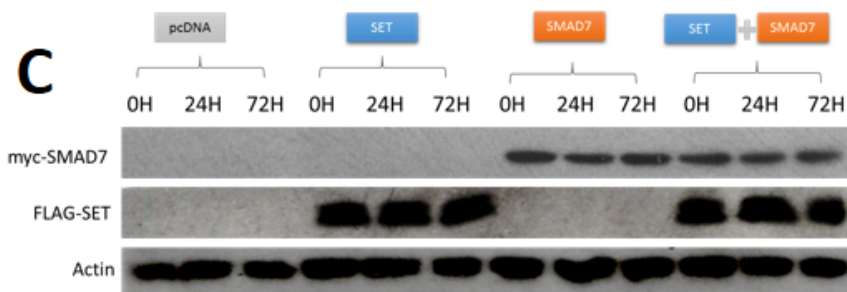
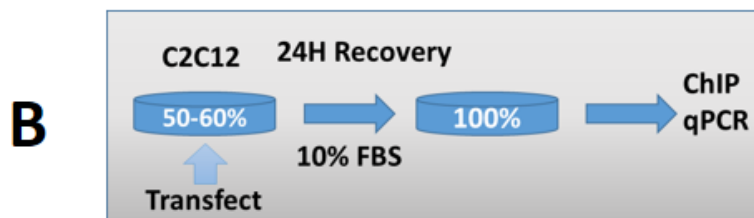
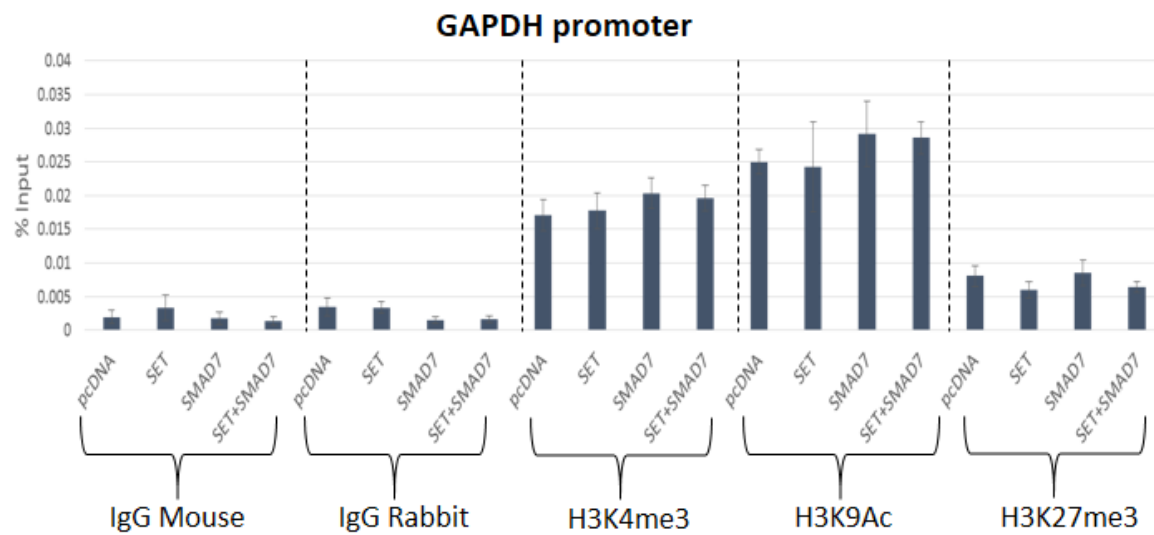
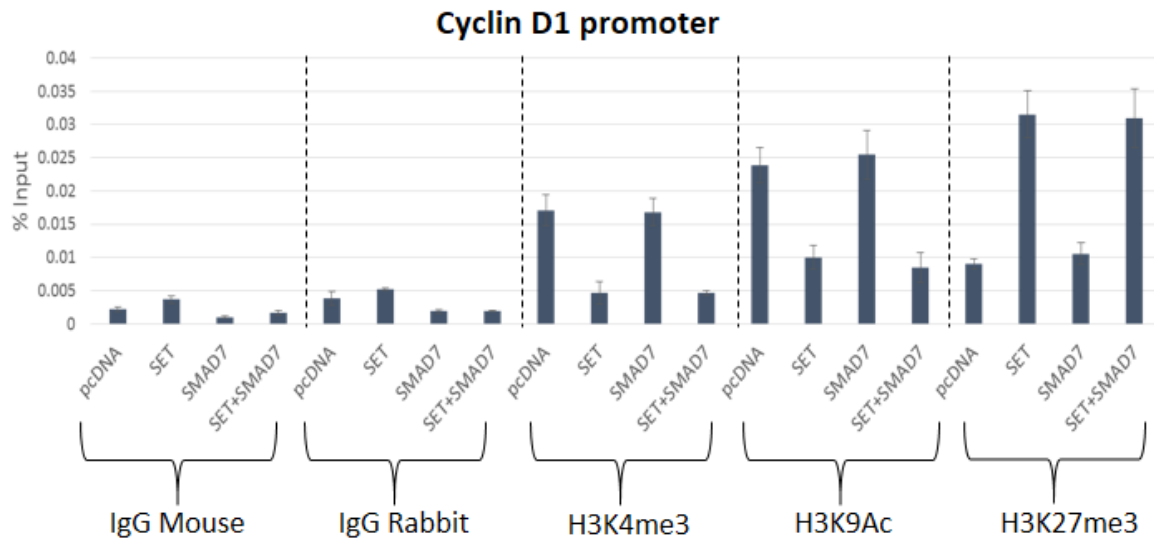


Figure 15: Epigenetic changes to the regulatory regions of *mck*, *myogenin*, *cyclin D1*, and *gapdh* in the presence of exogenous SET and/or Smad7. C2C12 cells were transfected with the stated constructs, left to recover for 24H in 10% FBS DMEM before crosslinking (4% paraformaldehyde), sonication, and ChIP with specified antibodies followed by qPCR using primers designed for regulatory regions of the genes (primer designs shown in **Supplementary Figure 3**). Rabbit IgG was used as ChIP control for the H3K4me3 and H3K27me3 antibodies while Mouse IgG was used as control for the H3K9Ac antibody. **A:** The Percent Input method was used to quantify the results displayed in the graph. Error bars represent SD values. **B:** Diagram showing summary of experimental design. **C:** Western blot analysis of total lysates from similarly transfected cells.

Discussion

Previous work from our group showed that exogenous Smad7 is able to promote myogenesis, leading to the upregulation of myogenic markers Myogenin, MCK, and MyHC. Although Smad7 is viewed as a potent inhibitor of the canonical TGF β /Smad3 pathway, it was unable to reverse TGF β 's inhibitory effect on myogenesis, suggesting a different role for Smad7 in myogenic cells. Smad7 was shown to physically interact with, and enhance MyoD's transcriptional properties (Kollias et al., 2006). A follow up study from our group revealed that nuclear Smad7 was able to promote myotube formation and upregulate myogenic markers even though Smad3 activity remained unchanged (Miyake et al., 2010). This further confirms the hypothesis that Smad7 promotes myogenesis via its nuclear role since depriving Smad7 of its cytoplasmic function did not reverse its effect on myogenesis. However, many questions remain unanswered about the regulation of Smad7 within the nucleus in contrast to the extensive research done on Smad7's cytoplasmic role. It is therefore essential to elucidate how Smad7's nuclear function is regulated within skeletal muscle, as its enhancement of myogenesis could have significant therapeutic potential.

A recent screen for Smad7 interacting proteins indicated an interaction between Smad7 and SET/TAF-I β , a nuclear proto-oncogene that is mainly known for its masking abilities of histone tails. SET, also known as 2PP2 α /IGAAD/TAF-I/12PP2 α /IPP2 α 2/PHAPII/TAF-I β , is a member of the Nuclear Assembly Protein 1 (NAP1) family (Park and Luger, 2006). SET was initially identified as the product of a gene that was fused to the *can* gene as a result of a translocation in a patient with acute undifferentiated leukemia (Von Lindern et al., 1992). Along with its histone chaperone properties, SET is a major subunit of the INHAT complex that masks histone proteins from modifications, i.e. P300 acetylation, by binding to the histone tails of

H3/H4 and blocking physical access of HATs, a mechanism referred to as ‘histone masking’ (Seo et al., 2001, Muto et al., 2007). SET was reported to stimulate transcription in an *in vitro* model adenovirus system by promoting structural changes around promoter regions by disrupting and remodeling the Ad core, making transcription and replication apparatus accessible to template DNA. A C-terminally truncated SET construct was completely inactive in this function, suggesting that the acidic C-terminus is required to stimulate transcription on chromatin (Matsumoto et al., 1999, Okuwaki and Nagata, 1998).

Many studies have revealed the involvement of SET in promoter specific transcription regulation. Using two-step ChIP experiments, Telese *et al.* show that SET forms a complex with the transcription factors Fe65, AICD, and Tip60 on the promoter region of the *KAI1* gene involved in cell adhesion. siRNA-mediated depletion of SET resulted in lower mRNA levels of KAI (Telese et al., 2005). Furthermore, Compagnone *et al.* show that SET (reported as StF-IT-1) forms a complex with the transcription factors COUP-TF, NGF-IB, and SF-1 on the *P450c17* gene which produces the crucial enzyme for synthesis of DHEA in the brain. The authors also report that SET’s developmental pattern of expression suggests it may participate in early ontogenesis of the nervous system, as well as the skeletal and hematopoietic systems (Compagnone et al., 2000). Kim *et al.* showed that SET is recruited to the promoter of the *atf3* gene, a member of CREB protein family of transcription factors. SET was shown to recognize and bind to PRC2-mediated H3K27me3 mark around the *atf3* promoter, further helping to repress the transcription of the *atf3* gene (Kim et al., 2012).

Ichijo *et al.* show that SET co-precipitated with glucocorticoid response elements (GREs) on the promoters of glucocorticoid receptor target genes in the absence of Dexamethasone. The DNA binding domain of the activated Glucocorticoid receptor was shown to bind to, and release SET

from glucocorticoid-responsive gene promotes, relieving transcriptional repression. Interestingly, the product of the fused *set-can* gene does not retain the same physiologic responsiveness to ligand bound GR, possibly contributing to the poor responsiveness of *set-can*-harboring leukemic cells to glucocorticoids(Ichijo et al., 2008).

Kim *et al.* reported that SET negatively regulates neuronal cell differentiation. The authors show that SET is expressed abundantly in neuronal tissues of *Drosophila* embryos during neuronal development, gradually reducing as differentiation proceeds. SET was shown to bind to the promoters of a subset of neuronal development markers, repressing the transcription of these genes by inhibiting acetylation of H4. siRNA mediated depletion of SET resulted in enhancement of neuronal cell differentiation, implicating SET as a negative regulator of neuronal development by targeting and ‘histone masking’ the promoters of the neuronal development markers(Kim et al., 2010).

SET was also shown to bind to, and repress p21 activity, a repressor of Cyclin B-CDK1, allowing cell cycle progression from G2 Phase into M phase (Canela et al., 2003). SET is also a potent inhibitor of Protein Phosphatase 2A (PP2A) as RNAi-mediated SET depletion resulted in increased activity and expression of PP2A and its target gene MMP-9 (Li et al., 1996). Moreover, SET also plays a role in Granzyme A-mediated apoptosis as it was shown to form a complex with, and regulate the activity of APE-1, a base excision repair endonuclease responsible for DNA repair (Fan et al., 2003).

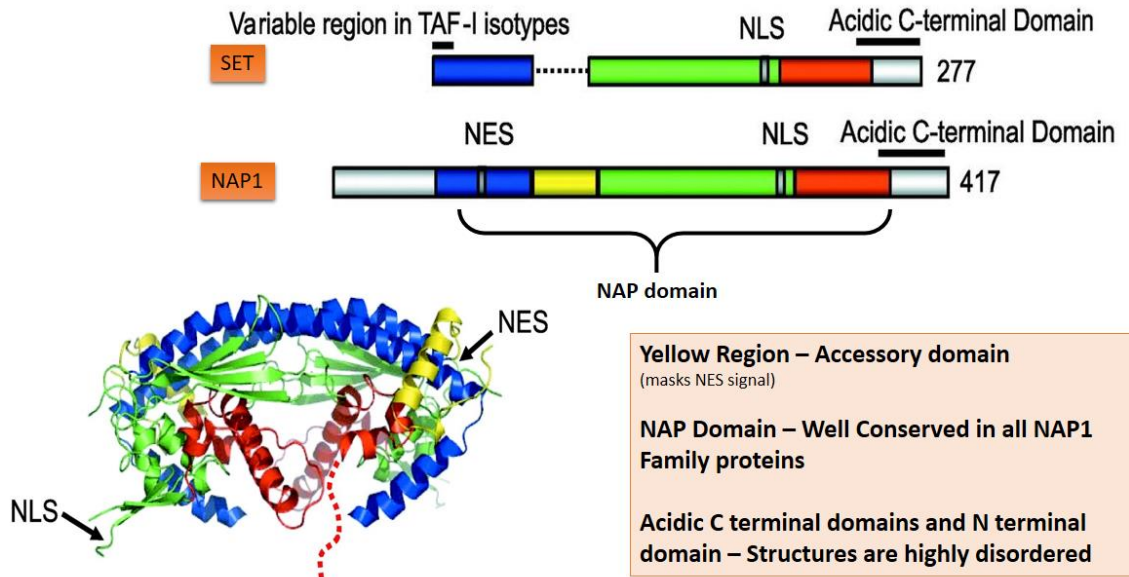


Figure 16: Comparison of the structure and functional domains of SET and NAP1. The NAP domain is required for histone protein binding while the highly acidic C terminal domain is required for binding and forming the INHAT complex.

The schematic diagram in **Figure 16** shows the structure and functional domains of SET. The NAP domain is highly conserved in all NAP1 family proteins and is required for histone protein binding. The highly acidic C-terminal domain of SET is highly disordered in structure and although it's not required for histone protein binding, it is a necessity for binding to and forming the INHAT complex. The SET protein exists as a dimer, forming a 'headphone' shape.

The focus of this study was the histone masking abilities of SET and its interaction with Smad7 in the context of myogenesis. The histone tail modifications on muscle specific genes are vital for the execution of the differentiation program since most transcription factors function on a chromatin template that depends on these modifications. Numerous covalent modifications that have been directly linked to regulating the transcriptional state of genes, such as H3K4me3 and H3K9Ac which have been associated with enhancing transcription, and H3K27me3 which is majorly reported to repress transcription of genes in proximity (Zhang et al., 2015).

Here we showed that the ectopic expression of SET resulted in an increase in total H3K27me3 levels in the cell lysates. Moreover, both promoters of *myog*, and *mck*, along with the upstream enhancer region of *myog* displayed increased levels of the H3K27me3 mark, resulting in lower myogenic markers at both the mRNA and protein level and inhibition of myogenesis. Ectopic expression of SET also resulted in increased levels of H3K27me3 mark at the promoter region of *cyclin D1* but no change in H3K27me3 levels was seen on the regulatory region of the housekeeping gene *gapdh*.

Although exogenous Smad7 alone did not display any change in the levels of H3K27me3, when introduced together with exogenous SET, Smad7 was able to not only reverse the effect of SET, it also led to a decrease in H3K27me3 levels compared to control. The lower levels of H3K27me3 corresponded with increased expression of MyoG and MCK at both the mRNA, and protein level. Ectopic expression of Smad7 alone did not change H3K27me3 levels on either *cyclin D1* or *gapdh* promoters. **(Figure 17)**

Ectopic expression of SET alone reduced levels of both transcription activating marks H3K4me3 and H3K9Ac on *mck* and *myog* promoters along with the *cyclin D1* promoter compared to control. In contrast, exogenous Smad7 alone was able to marginally increase H3K4me3 and H3K9ac levels on *mck* and *myog* promoters, but not on *cyclin D1* or *gapdh*. **(Figure 17)**

Remarkably, co-expression of exogenous SET and Smad7 led to higher levels of the transcriptionally activating marks H3K4me3 and H3K9Ac and lower H3K27me3 levels of the transcriptionally repressive mark on the *mck* and *myog* promoters than that of Smad7 alone. The introduction of SET and Smad7 resulted in a reversal of SET's repressing effect on the myogenic promoters, while at the same time, when introduced together with Smad7, SET was able to enhance Smad7's effect on activating the transcription of the *myog* and *mck* genes. The increase

in H3K4me3 and H3K9ac levels at the regulatory region of the *mck* and *myog* genes corresponded with increased expression at both the mRNA, and protein level. However, Smad7 was unable to modulate SET's repressive effects on the *cyclin D1* promoter when both exogenous Smad7 and SET are introduced, suggesting that Smad7 might only target SET at the regulatory regions of myogenic related genes. SET and Smad7 had no effect on the transcriptional activity of the *gapdh* promoter, even when introduced together. This could be due to absence of nucleosomes in proximity of the promoter of *gapdh* since it's always transcribed at constant high levels. (Figure 17)

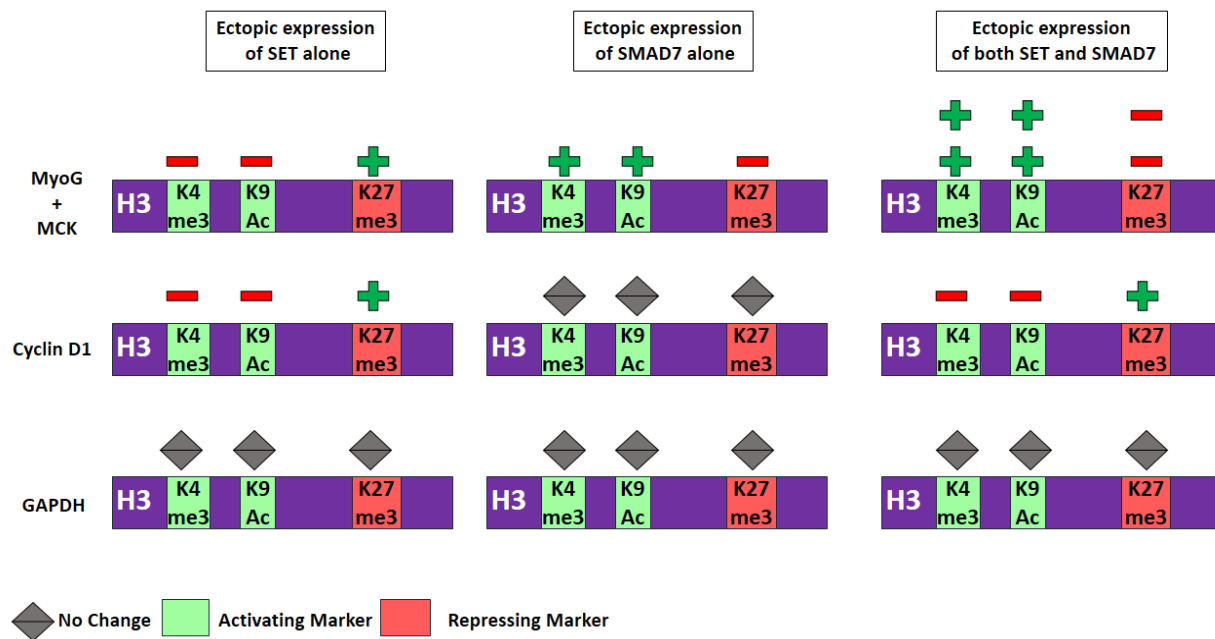


Figure 17: Schematic diagram summarizing the effects of exogenous SET and Smad7 to the histone marks around the regulatory regions of *mck*, *myogenin*, *cyclin D1*, and *gapdh* genes in the presence of exogenous SET and/or Smad7.

SET demonstrates an overall repressive effect on myogenesis by masking the histone 3 tail and preventing any transcriptionally activating modification on lysine residues K4 and K9, and SET concurrently promotes the repressive histone marker H3K27me3 at both the regulatory regions of *myog* and *mck*. This results in an inhibition of myotube formation and skeletal muscle differentiation. SET can therefore be seen as an antagonist of transcription of myogenic markers.

Here we show that Smad7's nuclear role displays contrasting effects to that of SET in the context of skeletal muscle differentiation. Ectopic expression of Smad7 resulted in an increase in transcriptionally activating histone marks and a decrease in repressive marks around the regulatory regions of myogenic marker genes *mck* and *myog* leading to an enhancement of myotube formation. Smad7 can therefore be seen as an agonist of transcription of myogenic markers and myogenesis. **(Figure 18)**

Surprisingly, ectopic expression of the antagonist SET and the agonist Smad7 together leads to an enhancement in myotube formation and an enrichment of the effects seen with exogenous Smad7 alone in terms of the upregulation of myogenic marker expression and the enhancement of myotube formation. This suggests that SET and Smad7 promote myogenesis when introduced together, even though SET is an antagonist of myogenesis when introduced alone. **(Figure 18)**

One plausible explanation is that Smad7 targets and inhibits the transcriptionally repressive effects of SET only in the regulatory regions of myogenic related genes, while at the same time, the surplus levels of the SET protein lead to further repression of non-myogenic related genes, possibly repressing anti-myogenic genes like the *cyclin D1* gene.

Our group previously reported that nuclear Smad7 is able to promote myogenesis by physically binding and enhancing MyoD's transcriptional activity which resulted in the upregulation of MyoG, MCK and MHC (Kollias et al., 2006). Smad7 could potentially form a complex with a number of different transcription factors including MyoD, and Smad7 could be recruited to SET at the regulatory regions of myogenic markers through MyoD (**Figure 18**). SET mediated repression of the cell cycle coupled with Smad7's antagonism of SET at myogenic genes results in a pronounced enhancement of myogenesis.

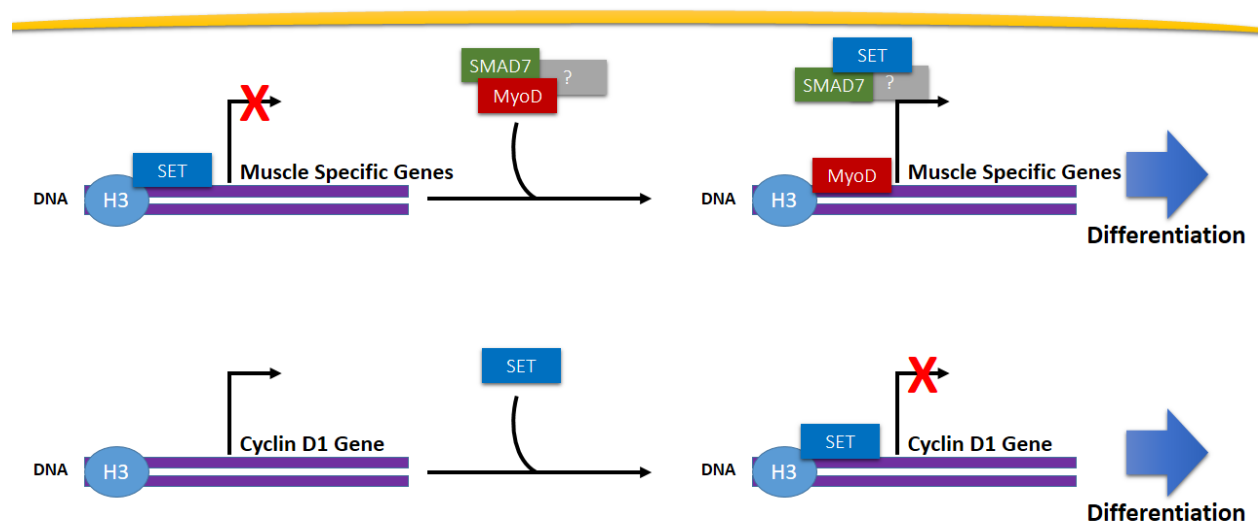


Figure 18: Epigenetic changes to muscle specific genes and the cyclin D1 gene when introducing ectopic SET and Smad7.

Summary and Future Direction

The results here show a transcriptional repressive role for SET in the context of myogenesis. SET was shown to induce epigenetic changes that result in transcriptional repression of myogenic related genes such as *mck* and *myogenin* as well as the cell cycle gene

cyclin D1. Smad7 interaction with SET near promoters of myogenic related genes leads to epigenetic changes that induce expression of those myogenic related genes. However, Smad7 has no effect on the transcriptional repressor role of SET on *cyclin D1*. SET mediated repression of the cell cycle coupled with Smad7's antagonism of SET at myogenic genes results in a pronounced enhancement of myogenesis.

The data here showcase a novel role for SET in the context of myogenesis as well as revealing alternative functions of Smad7 in the molecular control of myogenesis. This highlights the potential therapeutic use of Smad7 and SET to offset muscle wasting in a variety of muscle disorders. Future work would revolve around elucidation of the underlying mechanism in which Smad7 targets and antagonizes SET's repressive role on myogenic related genes but not cell cycle related genes such as *cyclin D1*. Performing ChIP-Seq analysis using histone mark specific antibodies on cell lysates with exogenous SET and Smad7 would provide a comprehensive overview of epigenetic changes when exogenous SET and Smad7 are present. This gives better understanding of the group of genes targeted for repression by SET and whether those genes are relieved from SET repression by Smad7.

To show direct recruitment of SET and Smad7 on the promoters affected, performing a two-step ChIP-Seq analysis using SET or Smad7 specific antibodies on cell lysates with exogenous SET and Smad7. However, there are currently no antibodies that can detect endogenous SET or Smad7 and attempts at using ChIP grade antibodies against tags of either exogenous protein have not been successful. Furthermore, it would be interesting to see how RNAi mediated depletion of endogenous SET would impact skeletal muscle differentiation given its importance in epigenetic control.

SET may have a major role in the changes to the epigenetic landscape that are essential for proper myogenesis to take place. More importantly, SET may play a major role in prenatal skeletal muscle formation and differentiation. SET may also be involved in satellite cell proliferation and differentiation postnatally. There are numerous studies that target SET as a therapeutic strategy for cancer (Farrell et al., 2014, Janghorban et al., 2014, Sobral et al., 2014, Mukhopadhyay et al., 2013). SET accumulation has been reported to exacerbate prostate, breast and pancreatic cancers and is being currently researched as a pharmaceutical target for cancer therapy. Here we have shown the *in vitro* role of SET in myogenesis and if SET plays a similar role in satellite cell proliferation and differentiation, it could serve as a potential therapeutic target for muscle regeneration and dystrophy.

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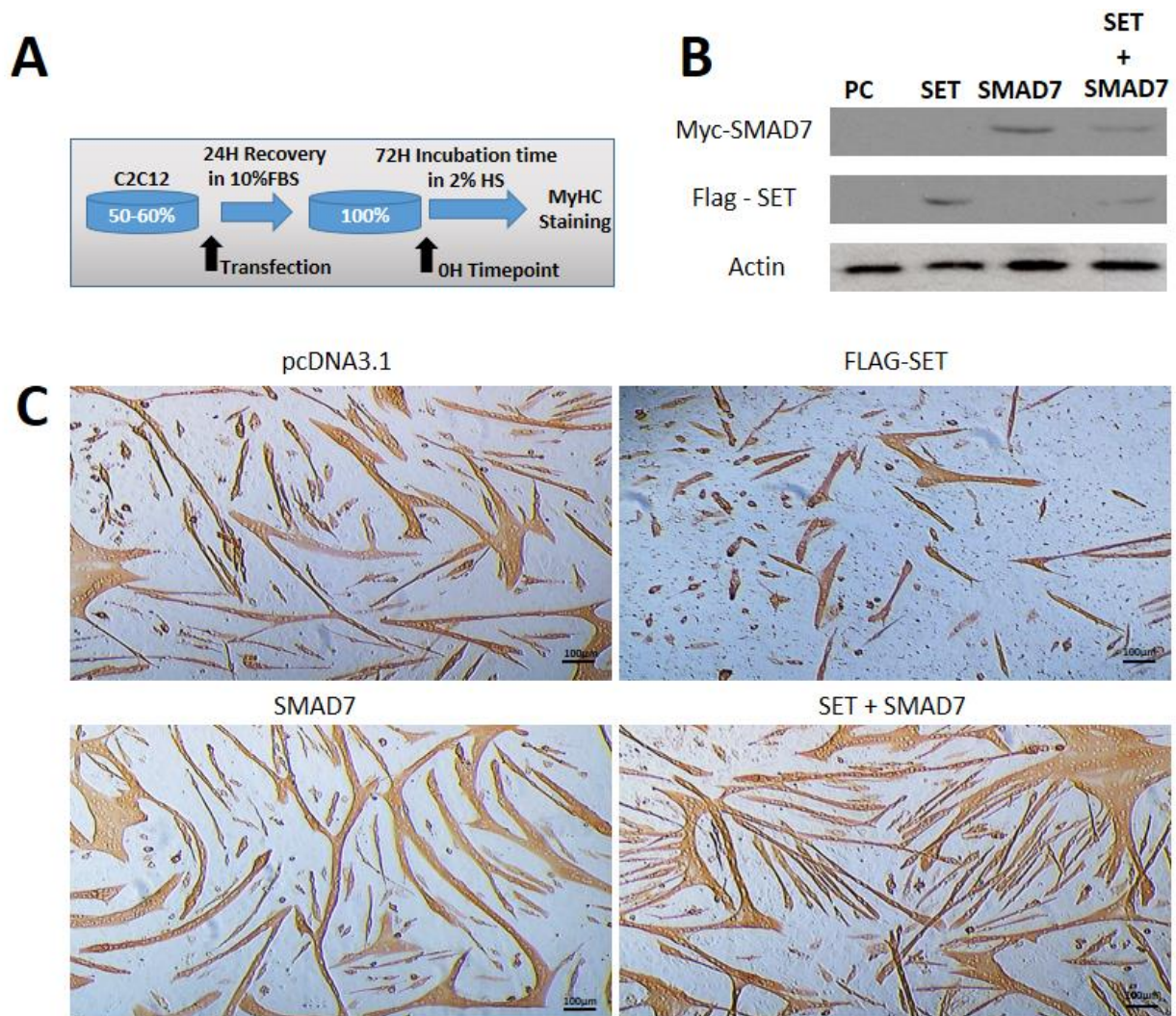
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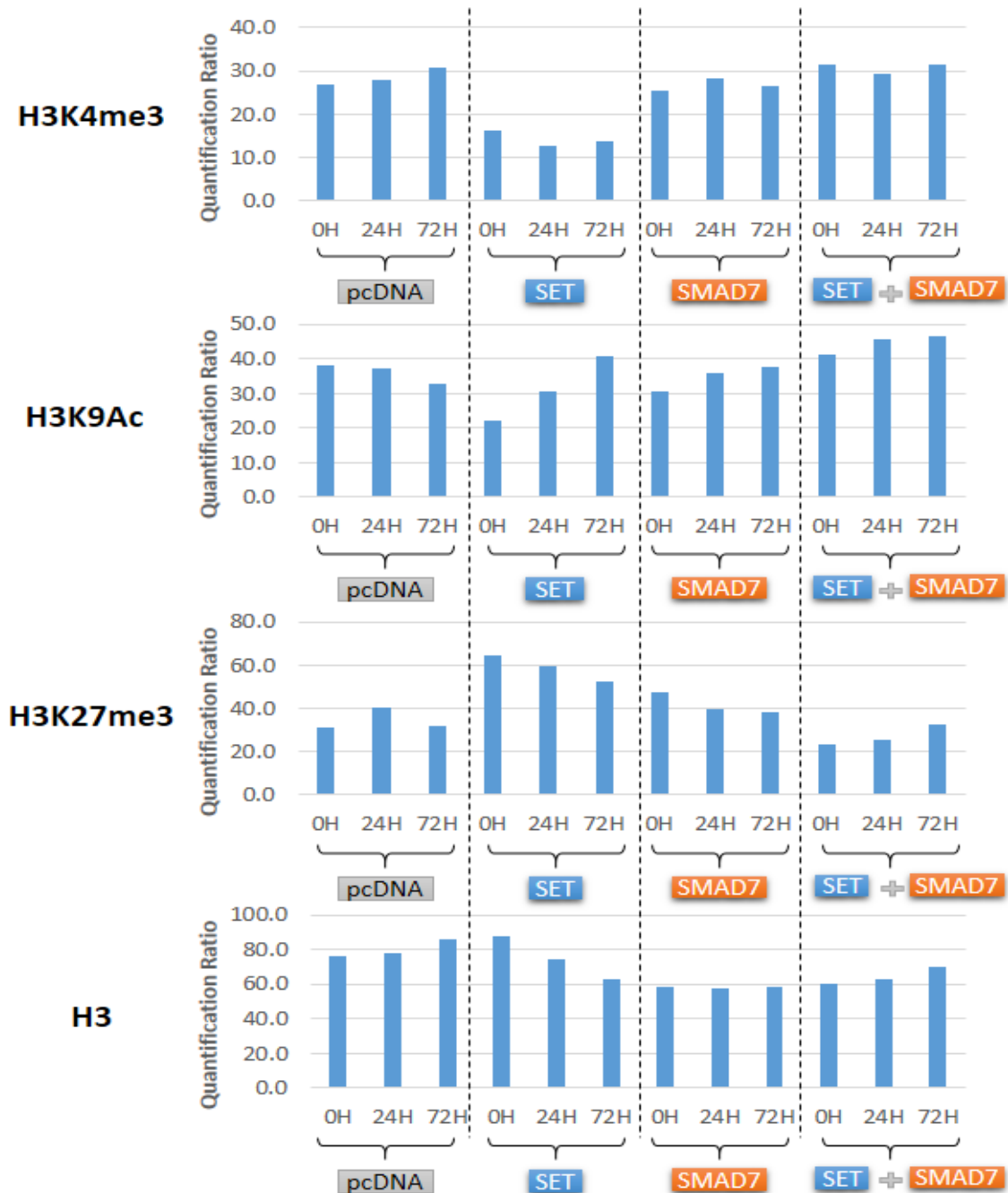
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Supplementary Figures

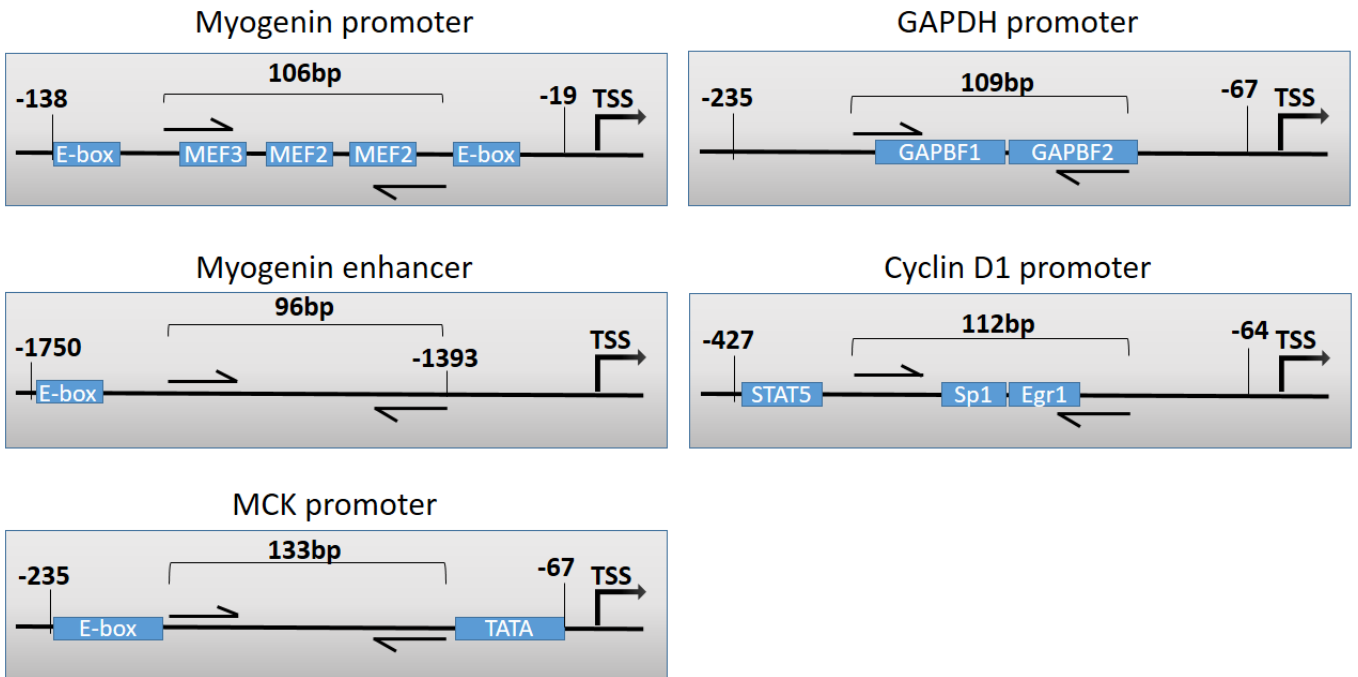


Supplementary Figure 1: Exogenous SET and Smad7 promote myogenesis while ectopic expression of SET alone leads to cell death. C2C12 cells were transfected with Flag-Set and/or Smad7, left to differentiate for 72H in low serum conditions (2% HS) followed by 4% paraformaldehyde fixation and immunostaining MyHC. **A:** Diagram showing summary of experimental design. **B:** Western blot analysis using lysates of similarly transfected cells **C:** MyHC staining showing level of myotube formation.



Supplementary Figure 2: Quantification of Western blots shown in Figure 14.

Quantification ratio is the Mean Grey Value (MGV) of each protein band divided by MGV of the corresponding lane's loading control. Quantification was done using ImageJ (1.8.77).



Supplementary Figure 3: Schematic diagram showing amplified regions in the ChIP experiments. Arrows depict the primers. TSS – Transcription Start Site.

Supplementary Tables

Supplementary Table S1: Primer Sequences for ChIP experiments

Gene	Forward Primer	Reverse Primer
Cyclin D1	5'-TTCGGGATGATTGGAATAGC-3'	5'-TGTGAGCTGGCTTCATTGAG-3'
GAPDH	5'- AGAGAGGGAGGAGGGGAAATG-3'	5'- AACAGGGAGGAGCAGAGAGCAC -3'
MCK	5'-ACCCGAGATGCCTGGTTAT-3'	5'-AGAGCGAGCTTCTCCTCCAT-3'
MyHC	5'-TAAGTCCAATATTAGAGATTCCAATTGAAGAT-3'	5'-GGTGTGGGGGAGCTGGCTTTTAG-3'
MyoG promoter	5'-CTGGAAACGTCTTGATGTGC-3'	5'-CCATTTAAACCCTCCCTGCT-3'
MyoG upstream	5'-CGGGTCCTGTCTGTTTACTACGTT-3'	5'- AATGCCTTCTGGCACTAGAACCGT-3'

Supplementary Table S2: Primer Sequences for RT-qPCR experiments

Gene	Forward Primer	Reverse Primer
Smad7	5'-TCCTGCTGTGCAAAGTGTTTC-3'	5'-TTGTTGTCCGAATTGAGCTG-3'
SET	5'-T TACTGACCATTCTGACGCA-3'	5'-CTGCCCTCTCCTTCTTCATCA-3'
MCK	5'-GGAGAAGGGAGGCAATATGAAG-3'	5'-GGTGCTCGTTCCACATGAA-3'
MyHC	5'-TGCCACATCTTGATCTGCTC-3'	5'-CTCGGCTTCAAGGAAAATTG-3'
MyoG	5'-CAGCTCCCTCAACCAGGAG-3'	5'-TGCAGGAGGCGCTGT-3'

Extended Materials and Methods

Western Blot Analysis Protocol

Wash Buffer

5mL of 10% tween 20

1L 1X TBS, final concentration is 0.5%

Transfer Buffer

100mL of 10X transfer buffer

100mL methanol

800mL of ddH₂O

10X Transfer Buffer

288g glycine

60.4g Tris base

1.8L dH₂O

Blocking Buffer

150mL 1X TBST Wash buffer

7.5g nonfat powder milk

Antibody Dilutions - 5mL

Antibody volume = Final volume X $\frac{1}{\text{dilution ratio}}$ X 1000 to Convert to μL

e.g 1:1000 dilution with final volume of 5mL

$(1/1000) \times 5 \times 1000 = 5\mu\text{L}$ of antibody

Add antibody volume to 0.05 X 5mL = 0.25g of powder milk

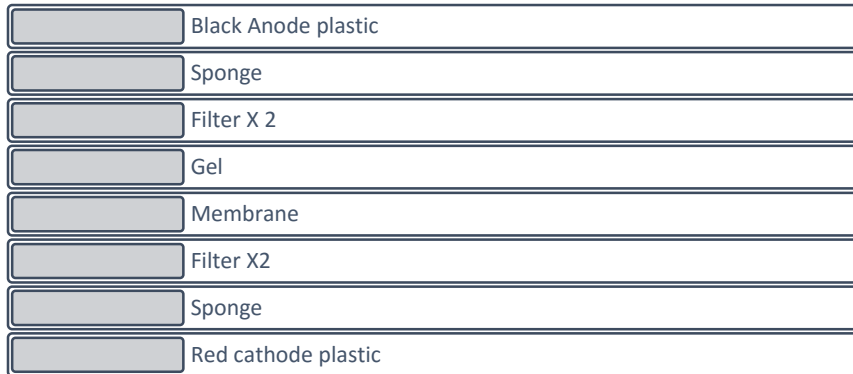
And 5mL of 1X TBST (also called wash buffer)

Western Sample preparation

1. Add a fifth of your lysate volume of 5X SDS-loading
2. Vortex and denature proteins at 95°C for 5min
3. Spin at max 14,000rpm at 4°C for 15min to pellet lipids

Western Blot overall Protocol

4. Make separating + stacking gel according to sheet in the lab, wait 1 hour at least to polymerize
5. Place gel inside the plastic cassette, place another on the other side to stop the buffer from leaking
6. Put in 1X Laemli buffer 1, wash wells with it as well
7. Load and run gel at 150volts (200volts for a quick run, this will produce a lot of heat)
8. Activate membrane in 95% methanol 5min prior to placing in sandwich
9. Create transfer sandwich



10. Wet all filter and sponges in 1X transfer buffer
11. Wash membrane and gel in transfer buffer
12. Put sandwich in transfer buffer, place ice container in the cassette, run at 100 volts for 1 Hour or 30 volts overnight
13. Wash membrane in wash buffer
14. Block membrane via blocking buffer for 1Hour
15. Put in sealed plastic bag with primary antibody, rock for 1 Hour or overnight in 4°C
16. Wash 3X for 5mins each
17. Put membrane in a new sealed plastic bag with secondary antibody, rock for 1 hour
18. Wash membrane 3X for 5mins each
19. Put equivalent amounts of ECL chemiluminescent bottles. Suggested volume is 500µl of each onto the membrane, dry and remove excess
20. Wait for 30 secs or 1 min, put membrane in between plastic sheet
21. Place film on top of membrane
22. Waiting times varies with strength of signal
23. Fix and Develop Film.

Calcium Phosphate (CaPO₄) Transfection

2X HeBS Solution pH 7.15

16.4g NaCl

11.9g HEPES

0.21g Na₂HPO₄

700ml ddH₂O

2.5M CaCl₂

36.76g CaCl₂—2 H₂O

100ml ddH₂O

CaPO₄ Overall Protocol

1. The day prior to transfection, seed C2C12 cells at 1×10^5 per plate if the harvest point is myoblast stage. If harvest point is myotube stage, seed cells at 2×10^5 per plate. Put fresh growth medium 10% FBS DMEM 3 hours prior to transfection.
2. For each plate being transfected, mix 25ug of total DNA mass to be transfected, bring volume up to 450ul using ddH₂O.
3. Add 500ml of cold 2X HeBS to a 14ml snap cap tube, place on vortexer, leave on to vortex at speeds 2 or 3. Don't press down hard on the vortexer since it will slow down the vortex speed.
4. Add 50ul of CaCl₂ to the DNA-H₂O mix, bringing total volume to 500ul. Mix well.
5. Slowly pipette droplets of the CaCl₂-DNA-H₂O mixture into the 2X HeBS in the 14ml scap cap tube while it's on the vortexer. Drop the DNA-CaCl₂ mix within 20seconds of

start time. Not too slow. Not too fast. Moderate speed gives the best transfection efficiency.

6. Incubate samples at RT for 25-30min, titrate 3-4 times, add mixture dropwise to the surface of the cells. Try to minimize impact force of the droplet by pipetting really close to the surface of the cells.
7. Incubate cells overnight in the incubator, don't leave the mixture on for over 18-20 hours.
8. Aspirate medium, wash cells twice with 1X PBS, put fresh 10% FBS DMEM. Leave cells to recover for 24H before harvesting or switching to 2% HS DMEM.

Co-Immunoprecipitation Assay

Wash Buffer

1mM EDTA (pH 8.0)

150mM NaCl

10mM Tris (pH 8.0)

0.1% NP40

NP-40 Lysis Buffer

0.5M PMSF

0.5M NaV

Pepstatin A(1ug/ul)

Leupeptin (10ug/ul)

Aprotinin (10ug/ul)

Overall Protocol

1. Wash cells with cold 1X PBS three times, place NP-40 lysis buffer on top of cells to be lysed during scraping and harvesting.
2. Collect cells/cell lysates and vortex for 10 second intervals every 5min for a total of 15min. Keep on ice.
3. Determine protein concentration via a Bradford Assay and use a total of 500ug of total protein for each sample. Bring volume of cell lysate up to 650ul for each sample using NP40 lysis buffer.
4. Add appropriate antibody or IgG (total 1ug) and incubate on rocker table at 4°C for minimum 1Hr.

5. Add 25ul of Protein A/G beads to the lysate-antibody mix. Incubate overnight on rocker at 4°C.
6. The following morning, pellet beads by centrifuging at 3600rpm for 3min at 4°C.
7. Aspirate supernatant, wash with 500ul of wash buffer three times, mixing and aspirating supernatant following centrifugation at 3600rpm for 3min at 4°C.
8. Add 25ul of 2X Laemmli loading buffer (with 2% β -mercaptoethanol), mix by vortex, followed by boiling at 95°C for 5min.
9. Spin samples at max 14,000rpm for 1min. Load 20ul of samples into each lane of SDS-PAGE gel.

MyHC Staining (6 well plates)

1. Aspirate media from the plates, wash three times using 1X PBS.
2. Incubate cells with 2ml of cold 90% methanol in ddH₂O for 5min at -20°C.
3. Wash three times using 1X PBS for 5 min.
4. Block in 2ml 5% non-fat skim milk in 1X PBS for 30min on the shaker at 37°C.
5. Incubate cells on the shaker with 1ml of 5% milk in 1X PBS with 1:20 MF-20 primary antibody for 2Hrs at RT.
6. Wash three times with 1X PBS, 5 minutes each, for a total of 15min on the shaker at RT.
7. Incubate with secondary antibody diluted 1:1000 in 5% milk in 1X PBS for 1 hour on the shaker at RT.
8. Wash three times with 1X PBS, 5 minutes each, for a total of 15min on the shaker at RT.
9. Mix 6mg of DAB in 10ml of 1X PBS, add 1:1000 30% hydrogen peroxide. Prepare fresh each time.
10. Incubate with DAB solution for 30min on the shaker at RT.
11. Wash three times with 1X PBS, 5 minutes each, for a total of 15min on the shaker at RT.
12. Wash two times with ddH₂O, 5 minutes each, for a total of 10min on the shaker at RT.

Reverse Transcription

1. Mix 1ul dNTP, 1ul oligodT, and 100-500ng of RNA (total RNA isolated using QIAGEN kit). Bring volume up to a total of 20ul using Rnase free water.
2. Heat at 65°C for 5min, put on ice.
3. Add 4ul of 5X First Strand Buffer and 2ul of 0.1M DTT
4. Incubate at 42°C for 2min.
5. Add superscript enzyme (0.2-0.5ul)
6. Mix by tapping, quick spin to collect all the solution at bottom of tube.
7. Run RT program - 42°C for 50min, 70°C for 15min
8. Store cDNA at -20°C.