Crosstalk between the planar cell polarity and hedgehog signaling pathways influences satellite cell fate

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This thesis is submitted as a partial fulfillment in the M.Sc. program in Cellular and Molecular Medicine

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Abstract

Our laboratory has identified two secreted proteins, Wnt7a and Sonic hedgehog (Shh), that regulate satellite cell (SC) fate, during muscle differentiation. While Wnt7a stimulates symmetric SC division through the planar cell polarity (PCP) pathway, Shh activates Myf5 expression in the committed SC following asymmetric division through cilia-mediated Hedgehog (Hh) signaling. Crosstalk between these pathways has been well characterized during development, and is likely to be conserved in muscle regeneration. Indeed, accumulating evidence suggests the PCP pathway influences primary cilia formation, an organelle required for proper Hh signal transduction. Here we show that Wnt7a treatment in primary myoblasts increases the presence of primary cilia. Additionally, using myofiber culture, we demonstrate that Wnt7a increases myogenin (MyoG) expression. Removal of primary cilia through a small interfering RNA (siRNA) targeted towards IFT88 impedes Wnt7a mediated MyoG expression, suggesting crosstalk between the PCP and Hh pathways facilitates muscle differentiation. Furthermore, through siRNA knockdown we have identified the downstream PCP effectors, Inturned and Fuzzy as the main candidates responsible for this crosstalk. Knockdown of either Inturned or Fuzzy impedes Wnt7a-mediated MyoG expression. Taken together our data demonstrates crosstalk between the PCP pathway and Hh signaling regulates the differentiation of SCs.

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List of Abbreviations

BBS - Bardet-Biedl syndrome
CamkII - calcium/calmodulin dependent kinase II
ChIP- Chromatin Immunoprecipitation
Cos2 - Costal 2
DMEM – Dulbecco's Modified Eagle Medium
DMD – Duchenne muscular dystrophy
DGC – Dystrophin Glycoprotein complex
Dvl2 - Disheveled 2
ECM - Extracellular matrix
EDL - Extensor digitorum longus
Fzd - Frizzled Receptor
Fu – Fused protein
Fuz- Fuzzy
GSK3 - Glycogen synthase kinase
Hh - Hedgehog
IFT- Intraflagellar Transport Protein
Intu - Inturned
LRP - Lipoprotein related receptor
MRFs - Myogenic Regulatory Factors
Myf5 - Myogenic Factor 5
MyoG - Myogenin
NFAT - Nuclear Family of Activated T-Cells
PFA – Paraformaldehyde
Ptch1 - Patched 1
PCP - Planar cell polarity
Shh - Sonic Hedgehog
siRNA – Short interfering RNA
Smo- Smoothened
SCs – Satellite Stem Cells
Vangl2 - Van-Gogh like 2
Wnt - Wingless
WT- Wild-type
YFP – Yellow Fluorescent Protein

Acknowledgements

I would like to acknowledge Dr. Michael Rudnicki for his supervision and providing me with the opportunity to work on this project. I would like to thank my thesis advisory committee, Dr. Jeff Dilworth and Dr. Stephanie Bennett, for their advice and guidance throughout this project. Furthermore, I would like to thank Dr. Caroline Brun for her guidance, mentorship and collaboration on this project. I would also like to recognize Hong Ming for his help with protocols and Jennifer Ritchie for her help with animal care. Finally, I would like to recognize my family, friends and especially George Freeman for their continuous support throughout this project.

Chapter 1: Introduction

1.1 Skeletal Muscle Regeneration

Skeletal muscle is one of the largest organs in the body as it comprises approximately 40% of total body weight (Frontera and Ochala 2015). In general, skeletal muscle holds vital roles in locomotion, posture, metabolism and respiration (Bushby et al. 2016). Skeletal muscle is widely recognized by its remarkable ability to regenerate and repair itself following injury (Otto et al. 2008; Von Maltzahn, J., Jones, A. E., Parks, R. J., & Rudnicki 2013; Dumont, et al. 2015). Muscle regeneration is a complicated, highly orchestrated process involving a group of transcription factors referred to as myogenic regulatory factors (MRFs) (Hernandez-Hernandez et al. 2017). MRFs are comprised of a series of basic helix loop helix proteins including myogenic factor 5 (Myf5), MyoD, myogenic regulatory factor 4 (MRF4) and myogenin (MyoG) (Stockdale 1992; Hernandez-Hernandez et al. 2017). During both embryonic myogenesis and adult muscle regeneration, the MRFs coordinate the commitment, proliferation and differentiation of muscle stem cells termed satellite cells.

First named because of their location between the sarcolemma membrane and basal lamina of myofibers, satellite cells (SCs) are now identified by their ubiquitous expression of the paired box transcription factor Pax7 (Seale et al. 2000). Deletion of *Pax7* leads to the loss of the SC pool, and subsequently impairs muscle regeneration, emphasizing its importance in SCs (Oustanina, Hause, and Braun

2004; Von Maltzahn et al 2013). In resting adult muscle, SCs reside in a quiescent state and comprise 2 to 5% of total muscle nuclei (Shenkman et al. 2010). Following injury, SCs become activated and re-enter the cell cycle. Proliferating muscle precursors, commonly referred to as myoblasts, are marked by the expression of Myf5 and/or MyoD (Seale et al. 2000; Stockdale 1992). Following several rounds of division, myoblasts down regulate *Pax7* and begin to express the differentiation markers MyoG and MRF4 (Seale et al. 2000; Stockdale 1992). Myoblasts that commit to differentiation permanently exit the cell cycle and undergo fusion to form new myofibers or repair existing ones (Seale et al. 2000; Stockdale 1992).

In addition to terminal differentiation, SCs also undergo self-renewal to replenish the stem cell pool (Kuang et al. 2007; Zammit et al. 2004). The self-renewing property of SCs is demonstrated during transplantation experiments in which a single SC contributed to the development of hundreds of new, multinucleated myofibers in addition to giving rise to a new resident SC population (Collins et al. 2005; Sacco et al. 2008). Overall, the ability of SCs to self-renew and differentiate is crucial for the maintenance of skeletal muscle.

1.1.2 Satellite Cell Division

SCs represent a heterogeneous population in which approximately 10% of SCs have never expressed *Myf5* (Kuang et al. 2007). *Myf5* negative (*Myf5*-) SCs are reported to repopulate the SC pool following engraftment into *Pax7*$^{-/-}$ muscle, suggesting the *Myf5*- SCs remain in a stem cell like state(Kuang et al. 2007). Conversely, the *Myf5* positive (*Myf5+*) population, terminally differentiate following

engraftment, representing a committed progenitor population (Kuang et al. 2007). Using an irreversible *ROSA26*^{CreYFP} reporter Kuang et al. (2007) demonstrated that proliferating SCs contribute to both *Myf5-* and *Myf5+* populations by dividing either symmetrically or asymmetrically (Kuang et al. 2007). A symmetric division occurs in a planar orientation with respect to the fiber and results in two *Myf5-* daughter stem cells. Conversely, an asymmetric division occurs in an apical basal orientation and gives rise to one *Myf5-* daughter stem cell, and one *Myf5+* committed progenitor (Figure 1) (Kuang et al. 2007).

In general, proper regulation of muscle regeneration is critical to maintain muscle homeostasis. Indeed, failed muscle regeneration is associated with a variety of diseases emphasizing the importance of the Pax7, MRFs and SC function(Shi and Garry 2006). For instance, malignant muscle tumors in rhabdomyosarcoma are attributed to myoblasts which have failed to exit the cell cycle and differentiate (Fu, et al. 2014; Koleva et al. 2005) . Additionally, failures in SC fate regulation during division have been associated with Duchenne muscular dystrophy (DMD)(Dumont, Wang, et al. 2015). These findings demonstrate the importance in understanding the mechanisms that regulate SCs and muscle regeneration.

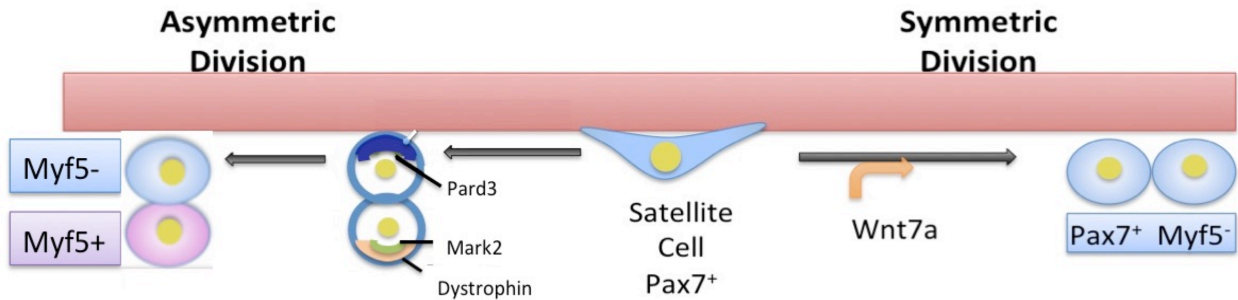

Figure 1: Asymmetric and Symmetric Satellite Stem Cell Divisions. Upon activation satellite cells may divide either symmetrically or asymmetrically. Symmetric divisions, occurring in a planar orientation, give rise to two identical Myf5- daughter cells. Addition of exogenous Wnt7a increases the proportion of these divisions. Conversely, an asymmetric division, which relies on dystrophin to establish the Mark2/Pard3 polarity complex, results in one Myf5- stem cell and one Myf5+ committed progenitor. Asymmetric divisions typically occur in an apical-basal orientation.

1.2 DMD and Dystrophin

Duchenne muscular dystrophy (DMD) is a progressive muscle wasting disease affecting 1 in every 3,500 boys (Durbeej and Campbell 2002; Chang, Chevalier, and Rudnicki 2016). DMD manifests during early years of life where children display difficulty when walking and completing other motor tasks. During preteen years, affected individuals completely lose ambulation. Ultimately, DMD patients die during the second or third decade of life, typically from respiratory failure(Durbeej and Campbell 2002). Current treatments for DMD are limited and typically involve the use of corticosteroids to delay the progression of symptoms by 2-5 years (Baxter 2010). Unfortunately, the administration of corticosteroids is accompanied by several undesirable side effects including bone density loss, weight gain, and hypertension (Baxter 2010).

The underlying cause of DMD is an X-linked loss-of-function mutation in the dystrophin gene *dmd*. Dystrophin is a large structural protein that is critical for the formation of the dystrophin associated glycoprotein complex (DGC)(Gumerson J. 2011). The DGC acts to stabilize muscle by connecting the actin cytoskeleton of muscle fibers to the extracellular matrix (Farini et al. 2009; Gumerson J. 2011). Mutations in other components of the DGC are associated with less severe muscular dystrophies such as limb-girdle muscular dystrophy (Gumerson J. 2011). Overall, lack of a complete DGC leaves muscle susceptible to contractile-induced damage resulting in multiple rounds of regeneration and eventually fibrotic infiltration.

Aside from its structural role within the DGC, dystrophin is also expressed in activated SCs, suggesting it has an alternative intracellular function (Dumont, Wang, et al. 2015). In 2015, Dumont and colleagues demonstrated that loss of dystrophin in the *mdx* mouse model of DMD impairs cellular polarity, and subsequently SC asymmetric divisions (Dumont et al. 2016). In SCs, dystrophin associates with the serine/threonine kinase, Mark2 (also known as *Par1b*). Mark2 phosphorylates the polarity factor Pard3, which consequently is polarized to the opposite side of the dividing cell to establish the Par polarity complex (Figure 1)(Dumont et al. 2016). This polarity complex is also known to regulate asymmetric divisions of neuroblasts and intestinal stem cells, highlighting its conserved role among various cell types (Goulas, Conder, and Knoblich 2012; Knoblich 2010). In SCs, knockdown of *Mark2* or *Pard3* significantly decreases the number of successful asymmetric divisions, and subsequently the number of committed myogenic progenitors (Dumont et al. 2016). Ultimately, these findings suggest that loss of dystrophin impairs the formation of the Par polarity complex required for successful asymmetric divisions. The loss of asymmetric division subsequently leads to a decrease in myogenic progenitors, thus impairing muscle regeneration in DMD. This study classified DMD as a stem cell disease and furthered our understanding of the mechanisms that regulate SCs.

Building our understanding of SC fate regulation is critical for developing SC targeted therapies for diseases such as DMD. A vast number of intrinsic and extrinsic factors have so far been identified to influence SC fate. These factors include but are not limited to cues from the immune system, extracellular matrix, paracrine and autocrine signaling (Dumont and Rudnicki 2016). Two families of

extracellular ligands, Wingless/Integrated (Wnt) and Hedgehog (Hh) have been of particular interest in SC fate regulation and are discussed in the following sections.

1.3 Wnt Signaling and Muscle Regeneration

Wnt signaling was first identified in *Drosophila melanogaster*, for its role in embryonic segmentation and polarization of the body axis during embryonic development (Le Grand et al. 2009; Rudnicki and Williams 2015). In vertebrates however, Wnt signaling is well known for its role in regulating tissue patterning and specification during development. In the paraxial mesoderm of a developing embryo, Wnt signaling activates various components of the myogenic lineage to promote muscle specification(Tajbakhsh et al. 1998). In adult tissue, Wnt signaling remains active to regulate various processes such as maintenance of cell polarity, proliferation and motility (Clevers 2006). In mammals, over twenty Wnt ligands have been identified to hold diverse signaling roles (Komiya and Habas 2008). Wnt ligands bind to a family of transmembrane Frizzled (Fzd) receptors in order to activate one of two classes of Wnt signaling, the canonical, β-catenin-dependent pathway or the non-canonical pathways. The non-canonical pathways are further subdivided into the planar cell polarity (PCP) pathway and the Wnt/Ca+ pathway (Clevers 2006; Rudnicki and Williams 2015; Le Grand et al. 2009; Wallingford and Mitchell 2011; Otto et al; 2012).

1.3.1 Canonical Wnt Pathways

Activation of the canonical pathway involves the formation of a protein complex comprised of Wnt bound to a Fzd receptor and either lipoprotein related receptor 5 or 6 (LRP5/6*)*(P. Satir, Pedersen, and Christensen 2010; Komiya and Habas 2008; Rudnicki and Williams 2015). The completion of this complex prevents the phosphorylation of β-catenin by glycogen synthase kinase-3 (GSK3), which in the absence of Wnt, targets β-catenin for ubiquitin-dependent proteolysis. Conversely, in the presence of a Wnt ligand, β-catenin is translocated into the nucleus where it promotes the transcription of target genes (Figure 2A) (P. Satir, Pedersen, and Christensen 2010; Komiya and Habas 2008; Rudnicki and Williams 2015).

The role of canonical Wnt signaling in postnatal muscle is currently debated. Some reports argue that overexpression of Wnt1 and Wnt3a stimulate canonical pathways to drive SC proliferation *in vitro* (Otto et al. 2008). Conversely, Le Grand and colleagues (2009) reported that *in vivo* overexpression of Wnt3a has no effect on SC numbers. Instead, Wnt3a overexpression was reported to be detrimental to muscle regeneration by promoting premature differentiation (Le Grand et al. 2009). However, more recent findings suggest that the timely silencing of β-catenin, rather than its activation is required for muscle regeneration (Murphy et al. 2014). These studies suggested that inhibition of Notch signaling was caused by the transient activation of the canonical Wnt pathway which was required to promote SC activation and differentiation (Murphy et al. 2014; Brack AS et al. 2007). In general, canonical Wnt signaling is thought to be an important influence in SC function.

However, the exact influence of the β-catenin-dependent pathway in the context of muscle regeneration requires further investigation.

1.3.2 Non-Canonical Wnt Pathways and Muscle Regeneration

In the Wnt/ Ca^+ signaling pathway, some Wnt ligand/Frizzled receptor complexes promote the release of Ca^+ from the endoplasmic reticulum through a G protein dependent process (Komiya and Habas 2008). The intracellular release Ca^+ activates several Ca^+ sensitive proteins including protein kinase C and calcium/calmodulin dependent kinase II (CamkII). CamkII has been shown to activate nuclear family of activated T-Cells (NFAT) transcription factors which mediate a variety of cell responses such as gastrulation during embryonic development, cell adhesion and inflammation (Komiya and Habas 2008; Calabria et al. 2009). In skeletal muscle, NFAT activation has been associated with specification of fiber type. However, the Wnt/ Ca^+ pathway has not yet been directly linked to SC cell function (Calabria et al. 2009). Due to its relatively new discovery, the Wnt/ Ca^+ pathway is the least studied Wnt pathway, and emerging evidence with regards to this pathway may be a future topic of discussion for DMD.

The PCP pathway has been further characterized compared to the Wnt/ Ca^+ pathway and has established roles in both embryonic development and adult tissue maintenance. During embryonic development the PCP pathway regulates the thinning of the mediolateral axis and the extension of the anterior-posterior axis in a process known as convergent extension (Komiya and Habas 2008). However in postnatal tissue, the PCP pathway is typically characterized by its regulation of the

actin cytoskeleton (P. Satir, Pedersen, and Christensen 2010). This is of particular interest in the study of stem cell diseases since cytoskeletal changes can affect cell polarity, which subsequently influences stem cell fate (Nance and Zallen 2011).

The PCP pathway has thus far been reported to be activated by Wnt4, Wnt5a, Wnt7a and Wnt11 (Komiya and Habas 2008). Binding of these Wnt ligands to their respective Fzd receptor engages a set of proteins known as the core PCP proteins. The core PCP proteins consist of Disheveled (Dvl), Prickled, Van Gogh-like (Vangl) and Cadherin, all of which are required for proper PCP signal transduction (Figure 2B)(Komiya and Habas 2008; Wang, Naturale, and Adler 2017). These proteins activate a series of downstream effectors that mediate cell type specific responses to the Wnt signal. The PCP effector proteins include Rac1, RhoA, Fuzzy, and Inturned, which co-ordinate cytoskeletal movements and cross-talk with other signaling pathways (Komiya and Habas 2008).

Of the PCP ligands, Wnt7a has been a key protein of interest in the study of muscle regeneration. Firstly, Wnt7a overexpression increases SC self-renewal by driving symmetric SC division (Figure 2B)(Le Grand et al. 2009). Silencing of the core PCP protein Vangl2 inhibits this increase in symmetric divisions, highlighting that Wnt7a acts through the PCP pathway to increase the regenerative potential of muscle (Le Grand et al. 2009). Secondly, addition of exogenous Wnt7a enhances the motility of committed muscle progenitors (Figure 2B) (Bentzinger et al. 2014). Prior to fusing with damaged myofibers, muscle progenitors must first migrate along the fiber to the site of injury. Thus, increasing progenitor motility may enhance muscle regeneration. Additionally, cell motility is important for engraftment assays since,

after injection into the recipient muscle, progenitors must migrate throughout the muscle to repopulate the progenitor pool. Indeed, exogenous Wnt7a treatment prior to transplantation is associated with increased SC engraftment, suggesting that Wnt7a could aid in the efficiency of stem cell based therapies for DMD (Bentzinger et al. 2014).

Aside from initiating the PCP pathway, Wnt7a binding to Fzd7 has been reported to also activate the Akt-mTOR pathway in fully differentiated myofibers. This activation of the Akt-mTOR pathway was associated with increased myofiber hypertrophy (von Maltzahn, Bentzinger, and Rudnicki 2012). Furthermore, Wnt7a treatment has been demonstrated to increase muscle strength and reduce mechanical muscle damage by promoting a shift towards a slow fiber type (Von Maltzahn, Renaud, et al. 2012). In general, the pleiotropic effects that Wnt7a has on muscle regeneration emphasize its potential to ameliorate DMD, making it a seminal candidate for muscle-focused therapies.

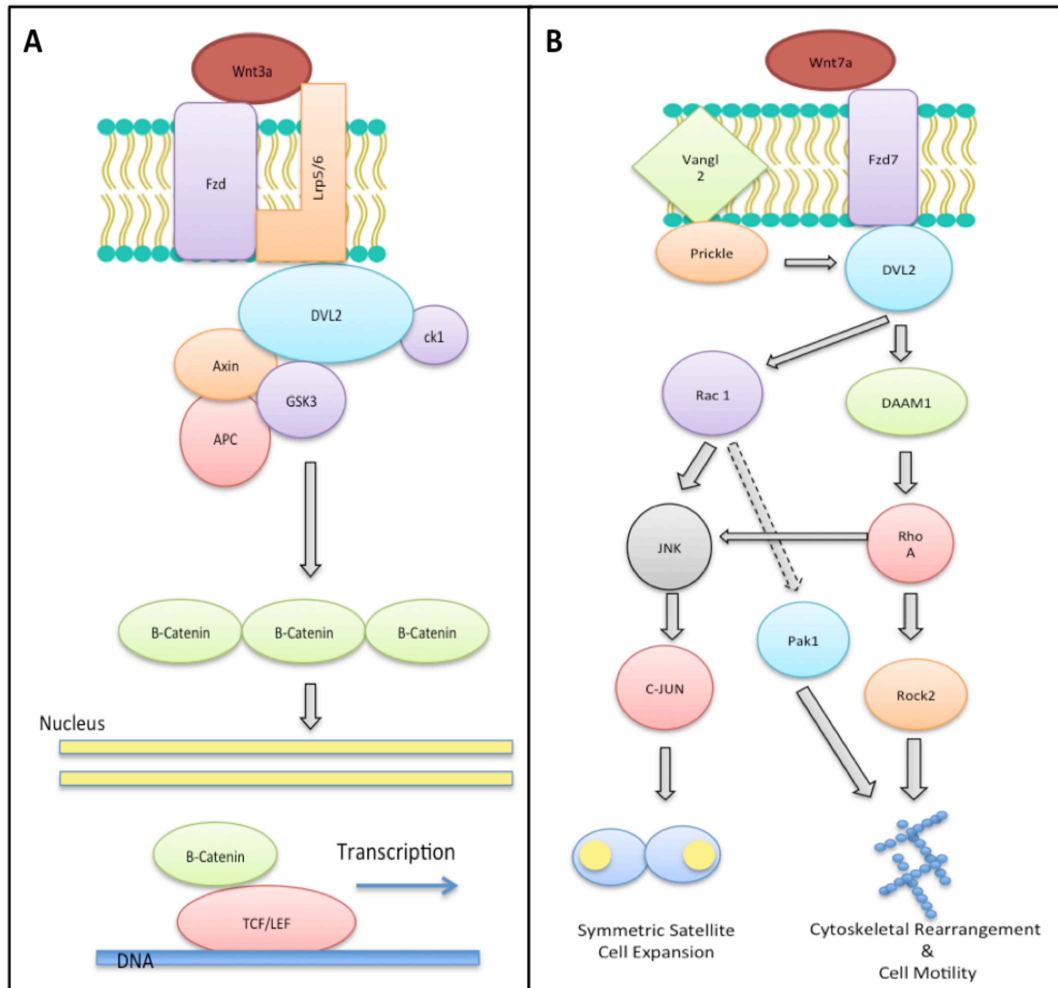

Figure 2: A schematic of the proposed Wnt signaling pathways. (A) Canonical Wnt signaling, upon the binding of the Wnt ligand the Fzd-LRP5/6 receptor complex recruits Dvl2 and Axin. This prevents the phosphorylation of β-catenin by glycogen synthase kinase-3 (GSK3) allowing for its accumulation in the cytoplasm and ultimately translocation into the nucleus where it promotes transcription of target genes. (B) The non-canonical planar cell polarity pathway also recruits Dvl2 in the presence the Wnt ligand. This engages a few possible cascades which ultimately results in cytoskeletal rearrangements or changes in cell polarity. The PCP pathway in muscle promotes cell motility and symmetric expansion of SCs.

1.3.3 Limitations of Wnt7a Treatment for DMD

Though our understanding of the relationship between the PCP pathway and muscle regeneration has vastly increased, a Wnt7a based therapy is not yet applicable to a clinical setting. Two conserved palmitoylation sites in the N-terminus of Wnt7a make the ligand highly hydrophobic and difficult to extract (Kurayoshi et al. 2007). These palmitoylation sites are argued to be crucial for proper Wnt secretion and function. However, a truncated form of Wnt7a consisting of 137 amino acids of the non-hydrophobic C-terminus was demonstrated to maintain full biological activity in skeletal muscle (Maltzahn et al. 2013). Conversely, studies focused on Wnt3a report that removal of the palmitoylation sites prevented both secretion of the protein and binding to the FZD/LRP6 complex (Hideyuki Komekado et al. 2007). However, non-canonical signaling does not require the activation of LRP6, which may allow for Wnt7a to maintain Fzd activation while lacking palmitoylation. Overall, a truncated Wnt7a seems to eliminate the challenges posed by the palmitoylation sites.

Further complications of Wnt7a treatment evolve from the non-specificity of Wnt signaling. The PCP pathway influences other processes such as postnatal neural development, emphasizing the requirement for a muscle specific delivery mechanism (Qiuhao Qu et al. 2013). Additionally, studies focused on Wnt7a treatment in muscle have not thoroughly considered crosstalk between the PCP pathway and other signaling cascades that influence muscle regeneration. Accumulating evidence supports the PCP pathway having influence on both Notch

and Hedgehog signaling cascades, which are involved in development and postnatal tissue maintenance (Taipale 2007; Capilla et al. 2012; Koleva et al. 2005). Given the conserved nature of these pathways, it is likely the PCP pathway influences other signaling cascades that are crucial for SC regulation.

1.4 Cilia Mediated Hedgehog Signaling

The primary cilium is an organelle which emanates from the cell surface during cell cycle arrest (Peter Satir, Pedersen, and Christensen 2010; Taipale 2007). Though primary cilia are commonly associated with cell motility, the primary cilium in many cell types such as myoblasts remains stationary. The primary cilia consists of two main components; a basal body, which acts as the base of the organelle, and the microtubule filaments, which allow for the projection of the primary cilia out of the cell (Peter Satir, Pedersen, and Christensen 2010; Taipale 2007). The primary cilia may be assembled and disassembled in a process known as ciliogenesis, which relies on the function of intraflagellar transport (IFT) proteins. Ciliogenesis is closely linked to cilia function and influences processes such as cilia mediated Hedgehog (Hh) signaling (Taipale 2007; Fu, Asp, Canter, and David 2014).

Primary cilia house many proteins required for Hedgehog signaling, another signaling cascade to have large developmental influences (Fu, Asp, Canter, and David 2014; May-Simera and Kelley 2012; Kopinke et al. 2017). During development, cilia-mediated Hh signaling is critical for dorsal-ventral patterning of tissues (Heydeck, Zeng, and Liu 2009; Zeng, Hoover, and Liu 2010). Disrupted Hh signaling is associated with several diseases known as ciliopathies, which result in

birth defects such as disrupted neural tube closure and polydactyly (Hildebrandt, Benzing, and Katsanis 2011; Wallingford and Mitchell 2011). In adult tissue, Hh signaling regulates the proliferation and differentiation of cells in various organs including the kidney, liver, brain, bone, and muscle (Fu, Asp, Canter, and Dynlacht 2014; Duprez, Fournier-Thibault, and Nicole Le Douarin 1998; Anne-Gaëlle Borycki et al. 1999).

Hh signaling is mediated by a family of secreted ligands comprising of Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh)(Anne-Gaëlle Borycki et al. 1999; Duprez, Fournier-Thibault, and Nicole Le Douarin 1998; Taipale 2007; Heydeck, Zeng, and Liu 2009). Binding of these ligands to the receptor patched1 (Ptch1) engages the Gli transcription factors (Gli1, Gli2 and Gli3) which are the primary proteins responsible for Hh signal transduction (Taipale 2007; Heydeck, Zeng, and Liu 2009). Of the Gli proteins Gli2 and Gli3 contain both transcriptional activation and repression domains. Though the repressor domain of only Gli3 is active, all Gli proteins including Gli1 have a functioning activation domain (Fu, Asp, Canter, and Dynlacht 2014; Taipale 2007; Peter Satir, Pedersen, and Christensen 2010). Both Gli2 and Gli3 may act independently of the other Gli proteins whereas Gli1 requires activation by either Gli2 or Gli3 (Fu, Asp, Canter, and Dynlacht 2014; Taipale 2007; Peter Satir, Pedersen, and Christensen 2010).

In the absence of the Hh ligand the activation domain of Gli3 is cleaved resulting in a truncated repressor form of the protein and the subsequent silencing of Hh target genes (Figure 3) (Taipale 2007; Heydeck, Zeng, and Liu 2009). Conversely, in its active state, binding of the Hh ligand to Ptch1, allows for the

translocation of Smoothened (Smo) into the ciliary membrane and the release of Fused (Fu) and Costal-2 (Cos2). This ultimately results in the release of the Gli transcription factors from the primary cilia allowing for their translocation into the nucleus via retrograde transport (Figure 3)(Peter Satir, Pedersen, and Christensen 2010; Taipale 2007).

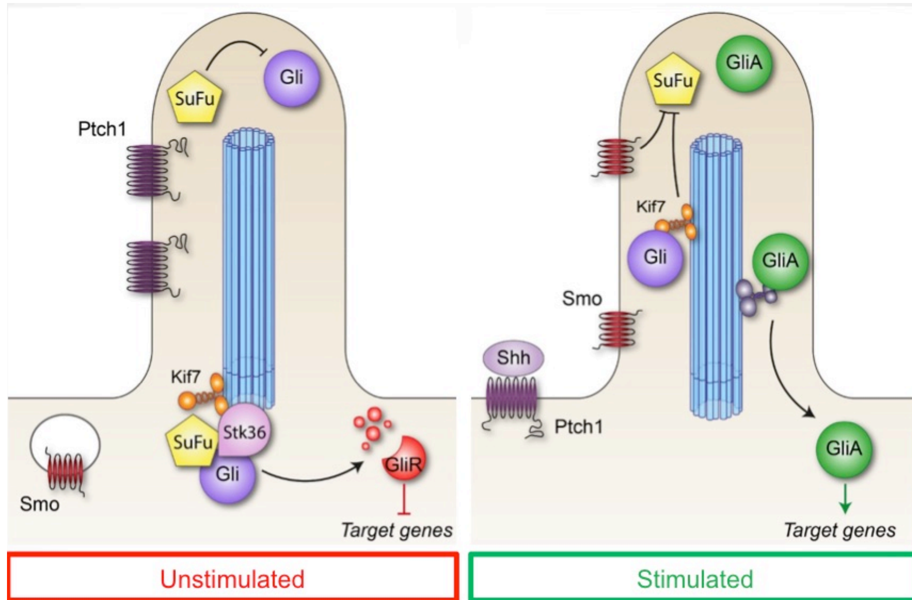

Figure 3: A schematic of cilia mediated Hh signaling. In the absence of the Hh ligand the translocation of Smo into the cilia membrane is inhibited, preventing the release of the activating Gli transcription factors into the nucleus. Additionally, Gli3 is processed into a repressor form, which enters the nucleus to silence Hh target genes (Left). When the Shh ligand binds to Ptch1, Smo is translocated into the ciliary membrane where it inhibits the Gli inhibitor, SuFu. This releases the Gli transcription factors, allowing them to travel via retrograde transport to the nucleus where they act to promote the expression of Hh target genes. Figure adapted from Caroline Brun; Unpublished

1.4.1 Hh Signaling and Muscle Regeneration

Hh signaling is known to regulate the commitment, proliferation and differentiation of myogenic cells during embryonic myogenesis (Fu, Asp, Canter, and David 2014; Kopinke et al. 2017; Anne-Gaëlle Borycki et al. 1999). Studies in developing mouse embryos provided evidence suggesting Gli2 and Gli3 bind directly to *Myf5*, committing cells to the muscle lineage and promoting myogenesis (Anne-Gaëlle Borycki et al. 1999). Specifically, Shh originating from the notochord was demonstrated to act on the medial-dorsal region of segments of paraxial mesoderm known as somites. During embryogenesis somites may be further compartmentalized. The medial-dorsal region referred to as the epaxial myotome, while the lateral- ventral region known as the hypaxial myotome. The epaxial myotome gives rise to all back muscles while the hypaxial myotome gives rise to the remaining muscle aside from those in the head (Tajbakhsh et al. 1998). Shh$^{-/-}$ mutant mice fail to express *Myf5* and *MyoD* and do not properly form the epaixal myotome (Anne-Gaëlle Borycki et al. 1999). Failure to form the epxaial myotome has lethal consequences, emphasizing the importance of Hh signaling during myogenesis.

Duprez and colleagues demonstrated that addition of exogenous Shh to chick embryo primary myoblasts increased bromodeoxyuridine (BrdU) incorporation into the DNA, suggesting that active Hh signaling increased myoblast proliferation. Similar effects were observed *in vitro* following Shh overexpression (Duprez, Fournier-Thibault, and Nicole Le Douarin 1998). Furthermore, loss of Hh signaling

prevents activation of the myogenic program in the early ventral limb (Jimmy Kuang-Hsien Hu et al. 2012). During later developmental stages, the same study found that Shh promotes the specification of slow muscle fibers (Jimmy Kuang-Hsien Hu et al. 2012). In general, Hh signaling holds critical roles in the activation and differentiation of muscle precursors during embryonic myogenesis.

Since muscle regeneration closely resembles embryonic myogenesis, it is likely that Hh signaling holds similar roles in both embryonic and postnatal muscle. Indeed, this holds true in the case of rhabdomyosarcoma in which the misregulation of Hh signaling not only affects proliferation of adult SCs, but also results in skeletal muscle differentiation defects (Fu, Asp, Canter, and Dynlacht 2014). Interestingly, this study demonstrated that primary cilia are transiently assembled and disassembled during muscle differentiation. Inhibition of primary cilia formation via siRNA targeted towards the intraflagellar transport protein *IFT88* (*siIFT88)*, severely disrupted Hh signaling and subsequently impaired myogenic differentiation (Fu, Asp, Canter, and David 2014). These observations were attributed to failed Gli3 cleavage, suggesting that proper Gli3 processing following transient ciliogenesis is required for myogenic differentiation (Fu, Asp, Canter, and Dynlacht 2014).

Additionally, Kopinke and colleagues demonstrated that knockdown of ciliogenesis via *siIFT88* in fibro/adipogenic precursors (FAPS), resulted in a shift from the Gli3 full-length activation form to the truncated repressor (Kopinke et al. 2017). This was associated with a decrease in Hh signaling reporters such as *Gli1* and Ptch1, further supporting the link between Hh signaling and ciliogenesis.

Conversely, myoblasts treated with Shh demonstrate increased proliferation which prevented their differentiation suggesting that silencing of the Hh pathway is required differentiation (Koleva et al. 2005). Taken together these studies suggest that the formation of the primary cilium is required for Gli3 processing which subsequently promotes myogenic differentiation. Preventing Gli3 processing by over stimulating Hh signaling inhibits myogenic differentiation emphasizing the importance of the Gli3 repressor. Overall, Hh signaling plays important roles in regulating the proliferation and differentiation of SCs highlighting Hh signaling as another therapeutic target for muscle focused therapies.

1.5 Links Between PCP and Shh Signaling

PCP and Hh signaling have a large influence on muscle regeneration. Therefore, understanding the crosstalk between these pathways is critical for the development of DMD targeted therapies. The first direct evidence of crosstalk between the PCP and Hh pathways was observed in the context of murine embryonic development (Heydeck, Zeng, and Liu 2009; Zeng, Hoover, and Liu 2010). These studies demonstrated that loss of either PCP effector, Fuzzy (Fuz) or Inturned (Intu), resulted in decreased cilia numbers, impaired Gli3 processing and mislocalization of Hh receptors, Ptch1 and Smo (Zeng, Hoover, and Liu 2010; Heydeck, Zeng, and Liu 2009). These observations were associated with embryonic defects such as failed neural tube closure and polydactyly, which is consistent with aberrant Hh signaling (Heydeck, Zeng, and Liu 2009; Zeng, Hoover, and Liu 2010; Wallingford and Mitchell 2011). Overall, these studies suggested that proper PCP

signal transduction is required for ciliogenesis. Additional evidence suggesting PCP proteins influence cilia formation arise from observations that the core PCP component, Dvl2, localizes at the basal body of primary cilia (Sánchez and Dynlacht 2016). Zilber et al (2013) demonstrated that Dvl2 and Fuz localization at the basal body was required for recruitment and activation of *RAB8*, a protein required to sustain cilia formation in mammalian cells (Zilber et al. 2013). Therefore, accumulating evidence suggests that PCP proteins hold influence over primary cilia formation.

It is important to note that crosstalk between the PCP pathway and Hh signaling is likely not unidirectional. Though the influence of cilia on PCP signaling requires further investigation, reports from studying Bardet-Biedl syndrome (BBS) proteins support the notion that Hh signaling and primary cilia may also influence PCP signaling. *BBS* proteins localize at the base of the primary cilium, and loss of these proteins results in ciliary defects (Ross et al. 2005; Gerdes et al. 2007). Gerdes and colleagues demonstrated in zebrafish that loss of *BBS1, BBS4* or *BBS6* resulted in convergent extension defects, processes highly regulated by the PCP pathway (Gerdes et al. 2007). These observations were associated with aberrant canonical and non-canonical Wnt signaling, suggesting that ciliary proteins are required for proper Wnt signaling. It is important to note that Hh signaling has been reported to differ between zebrafish and mammalian models. However, similar studies completed in murine models confirmed this link (Ross et al. 2005).

In this study, we first confirm the influence of Wnt7a on satellite cell proliferation and differentiation. We then look at the potential for crosstalk between

the Wnt-activated PCP pathway and Hh signaling during muscle regeneration. Finally, we propose that Wnt7a activation of the PCP pathway promotes cilia formation via Inturned and Fuzzy, which allows for Gli3 processing resulting in increased SC differentiation.

Chapter 2: Materials and Methods

2.1 Cell Culture

Primary myoblasts were obtained from hindlimb muscles of 6 week old C57bl/6 or C57bl/10 mice via magnetic activated cell sorting (MACS) as previously described(Sincennes, Wang, and Rudnicki 2017). Myoblasts were cultivated in Ham's-F10 medium 1X (Wisent) supplemented with 20% Fetal bovine serum (FBS)(Gibco), 2.5ng/ml of beta fibroblast growth factor (Millipore) and 1% penicillin/streptomycin (Wisent). Myoblasts were cultured on plates coated for 1 hour (h) with 0.01% collagen (VWR) and allowed to dry overnight. Cells were cultured at 37°C in a 5% CO_2 incubator.

For differentiation assays myoblasts were seeded 150,000 cells per well in a collagen coated 6-well dish. Once myoblast confluency reached ~80% (approximately 24h after seeding) cells were differentiated by switching to a medium comprised of Hams-F10: Dulbecco's modified eagle's medium (DMEM) (1:1)(Wisent), supplemented with 5% horse serum (Life Technologies) and 1% penicillin/streptomycin (Wisent). The introduction to differentiation conditions was considered as time 0, and samples were harvested or fixed at 24h intervals over a

72h period. For Wnt7a treatments recombinant Wnt7a (eBioscience) was resuspended in PBS and added to the medium for a final concentration of 100 ng/ml. Wnt7a and media was replenished daily, while in non-treated groups the medium was changed.

2.2 Immunohistochemistry

Cells or myofibers were washed once with PBS to remove any debris from culture then fixed for 10 minutes in 4% paraformaldehyde (PFA) diluted in PBS. Samples were washed three times for 5 min with PBS and then permeabilized for 10-15 min in a buffer comprised of 0.1M glycine (Fisher) and 0.1% Triton X-100 (Sigma-Aldrich) in PBS. Afterwards, samples were blocked with PBS containing 5% horse serum, 2% BSA (Fisher), and 0.1% TritonX-100 for 1-2 hours. Samples were incubated in primary antibodies diluted in blocking solution overnight at 4°C. Primary antibodies utilized are as listed in Table 1. Samples were washed 3 times for 5 min with PBS prior to a 1h incubation with fluorophore conjugated secondary antibodies diluted in blocking solution. This was followed by 3 more washes for 5 min with PBS prior to a 10 min incubation with Hoechst dye (Millipore) in PBS at a concentration of 5μg/ml. Cells were imaged in PBS while myofibers were mounted onto glass slides using Fluoroshield mounting medium (Sigma).

Table 1: List of Primary Antibodies Utilized for Immunostaining

Antigen	Supplier	Ig Type	Dilution
Pax 7	DSHB	Mouse IgG1	Undiluted
GFP	Abcam ltd.	Chicken IgG	1:1000
Pericentrin	Abcam ltd.	Rabbit IgG	1:500
α Acetylated Tubulin	Abcam ltd.	Mouse IgG2b	1:500
Myogenin (M225)	Santa Cruz	Rabbit IgG	1:500
Myogenin (F5D)	Santa Cruz	Mouse IgG1	1:500
Arl13b	Abcam ltd.	Mouse IgG2a	1:500
mCherry	Abcam ltd.	Mouse IgG	1:500
Syndecan 4 (H-17)	Santa Cruz	Goat IgG	1:100

2.3 Mice and Animal Care

The mouse strains utilized in this study are as follows: *mdx* and wildtype, *Myf5-Cre:ROSA26-YFP*$^{+/-}$ and *Arl13b/mCherry*. All mice strains were on a C57BL/10J background. Both male and female mice, 8-10 weeks of age, were utilized for all experiments unless otherwise stated. All experiments were conducted in accordance with the University of Ottawa guidelines for animal care.

2.4 Myofiber Isolation

Extensor digitorum longus (EDL) myofibers were dissected from sacrificed mice by first removing the skin, fascia and *tibialis anterior* without touching the EDL or its tendons. After moving any tissue covering the tendons of the EDL, both tendons were clipped and the EDL was slowly removed (Brun, Wang, and Rudnicki 2018). Once isolated each EDL was incubated in pre-warmed 0.2% Collagenase

Type I (Worthington) dissolved in DMEM (Gibco) supplemented with 1% penicillin/streptomycin, at 37°C for 1h for WT and 1.5h for *mdx*. All dishes utilized were coated in FBS for 5 minutes and allowed dry for 15 minutes to prevent attachment of myofibers. The EDL was triturated in a 10cm dish containing 10ml of DMEM to release myofibers from the tendon. Fibers were then washed 3 times by moving into a 60mm dish with 4ml of fresh DMEM. A 5-10 min recovery period at 37°C was allocated between each wash. Prior to culture, myofibers were allowed 1h to recover in the final wash at 37°C.

Myofibers were cultured in 6-well dishes containing 2ml of medium comprised of 1% Chick embryo Extract, 20% FBS, in DMEM supplemented with 1g/L Glucose, L-Glutamine, and 110mg/ml sodium pyruvate (Gibco). For Wnt7a treatments recombinant Wnt7a suspended in PBS was added to the culture medium (100 ng/ml, eBioscience). Myofibers were cultured at 37°C in a 5% CO_2 incubator.

2.5 Myofiber siRNA Transfection

Prior to use on fibers siRNAs were validated on myoblasts and knockdown was confirmed through RT-qPCR (Figure 4). Myoblasts were seeded in a 6-well dish at a density of 10,000 cells/cm^2 and cultured for 24h prior to the first transfection. Myoblasts were transfected with 5nM of each siRNA using RNAiMax (Invitrogen) diluted in Opti-MEM (Life Technologies), according to manufacturer's protocols. A second transfection was applied to fresh medium 24h after the first treatment. Myoblasts were then lysed 24h after the second siRNA treatment for a total of a 48h

treatment period. Some myoblasts were also treated with a scrambled siRNA (*siCntl*) at the same concentrations for comparison.

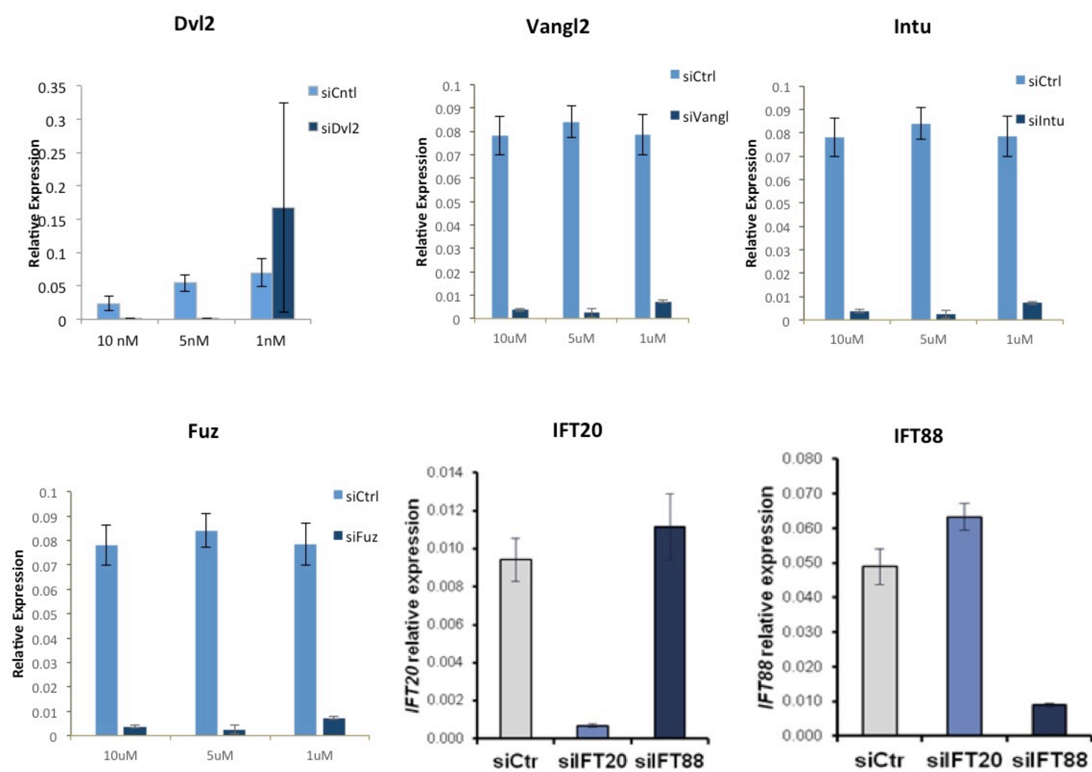

Figure 4: Validation of siRNAs via RT-qPCR on treated myoblasts. *siIFT20* and *siIFT88* were provided and validated by Caroline Brun.

Prior to transfection, myofibers were isolated as stated above and cultured in a 12 well dish with 2ml of culture medium for 4 hours prior to transfection. Myofibers were transfected with 5nM of each siRNA using RNAiMax (Invitrogen) diluted in Opti-MEM (Life Technologies), according to manufacturer's protocols. After 12 hours of incubation the transfection was repeated. After 6 hours of incubation in the second transfection the myofiber culture medium was replaced with fresh medium. After completion of the second transfection fiber culture, the fixation was carried out as stated above. The following siRNAs were used in this study: *siCntl*, *siIFT88, siIFT20*, *siDvl2*, *siIntu siFuz* (designed by IDT).

2.5 Image Acquisition

Images were attained at room temperature using an inverted fluorescence microscope (Zen observer D.1) and the Zen Blue imaging software. Images were composed in Fiji (ImageJ).

2.7 Quantitative Real-Time PCR

RNA was isolated with a commercial kit (NucleoSpin RNA II, Macherey-Nagel) as per manufacturers protocol. Reverse transcription was then carried out using a mix of oligoDTs, random primers and superscript III (Invitrogen). qPCR analysis was performed using SYBR green (Bio-Rad), and CFX384 real-time PCR detection system (Bio-Rad). Data was analyzed using the delta-delta CT method and represented as fold change in comparison to controls. Data represents three

separate biological replicates and expressed as means ± SEM. Primer sequences utilized are listed in Table 2.

Table 2: List of Primers Utilized for RT- qPCR

Target Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
Dishevelled2	CGTCACAGATTCCACAATGTCT	TCGTTGCTCATGTTCTCAAAGT
Fuzzy	AGAAAGAATTGAGGGCCAGC	ATGACACAGTCCACACACTG
Gli1	AGGGGTAGGTGAAGCTCAAT	AGGGGTAGGTGAAGCTCAAT
Gli3	TAGCTTCGACCTTCAGACCA	AGGGGTAGGTGAAGCTCAAT
Inturned	CTGAGACCTGTGAAAGAA	CTGAGACTGAGTCGTGAAA
Myf5	AGGGGTAGGTGAAGCTCAAT	AGGGGTAGGTGAAGCTCAAT
Myogenin	AGGGGTAGGTGAAGCTCAAT	AGGGGTAGGTGAAGCTCAAT
Van-Gogh2	CCCCAGTTCACACTCCAAGGT	ACTTGGGCAGGTTGAGGAG

2.8 Protein Extraction and Western Blotting

Protein extraction was performed on cells that were lysed using 200μl of RIPA buffer (ThermoScientific) supplemented with protease inhibitors (Roche), scraped off culture plates and placed into a 1.5ml-Eppendorf. Samples were incubated on ice for 1h then centrifuged at 4°C for 20min at 12 000rpm to remove any debris. The supernatant was collected and placed into a new Eppendorf. Protein concentration was estimated utilizing spectrometry and Bradford reagent (BioRad Lifesciences).

20μg of each protein lysate was added to 6μl of 6x Laemmli loading buffer. Samples were resolved on a 10% SDS-PAGE gel and transferred onto a PVDF membrane. The membrane was blocked for minimum 1h with 5% milk diluted in TBST then incubated overnight at 4°C in primary antibodies for Myogenin (F5D; Santa Cruz; 1:500 dilution) and Tubulin (T9026; Sigma; 1:5000 dilution) diluted in blocking solution. Membranes were washed 4 times for 10min in TBST prior to the addition of a HRP light chain specific secondary antibody (Jackson Immunohistochemistry) and used at a 1:5000 dilution in blocking solution.

Following a1h incubation at room temperature in the secondary antibody, membranes were washed another 4 times (10 min each). Membranes were then exposed using ECL (GE Life technologies).

2.9 Statistical Analysis

Statistical analysis was preformed using the GraphPad Prism Software (GraphPad Software, La Jolla, CA, USA). Unpaired student's t-tests were performed using the cut off value of $p < 0.05$. All experiments used a minimum of three biological replicates and error bars are represented as ± standard error of the mean (SEM)

Chapter 3: Results

3.1 Wnt7a Promotes Symmetric Expansion of Satellite Cells and Myogenic Differentiation

Wnt7a has been established to promote symmetric satellite cell divisions, thus we utilized *Myf5-Cre:R26R-YFP* reporter mice to isolate *extensor digitorum longus* (EDL) myofibers. The *Myf5-Cre:R26R-YFP* mouse model is a Cre-recombinase reporter system developed to label cells that have expressed *Myf5* with the irreversible expression of yellow fluorescent protein (YFP)(Kuang et al. 2007). This system allows the proportion of asymmetric and symmetric divisions to be quantified following the first round of SC division.

In contrast to asymmetric divisions, symmetric divisions occur in a planar orientation with respect to the fiber. Therefore, *Myf5-Cre:R26R-YFP* myofibers were treated with 100ng/ml of Wnt7a, and fixed at 36h of culture. This is the time at which centrosomes migrate to opposite poles of the activated SC, defining the orientation of division (Brun, Wang, and Rudnicki 2018). To analyze the orientation of division myofibers were stained for pericentrin and α-acetylated tubulin to identify centrosomes and mitotic spindles, respectively. The angle of division was measured in each dividing cell by measuring the angle between the line of the centrosomes and the myofiber. Centrosome pairs measured greater than 45° were considered an apical-basal division, while those less than 45° were considered to be in a planar orientation.

In untreated wild-type (WT) fibers, 65% centrosome pairs were aligned in a symmetric orientation, while in the presence of Wnt7a this increased to 79% (Figure 5A). Wnt7a treated *mdx* fibers demonstrated a similar increase in symmetric divisions, despite starting with a higher proportion of cells in a planar orientation compared to WT (72% Non-treated, and 84% treated) (Figure 5A). These findings are consistent with the decreased ability of *mdx* SCs to divide asymmetrically (Dumont et al. 2016). Overall, these results demonstrate that Wnt7a treatment promotes SCs to divide in a planar orientation, a characteristic of symmetric divisions (Le Grand et al. 2009).

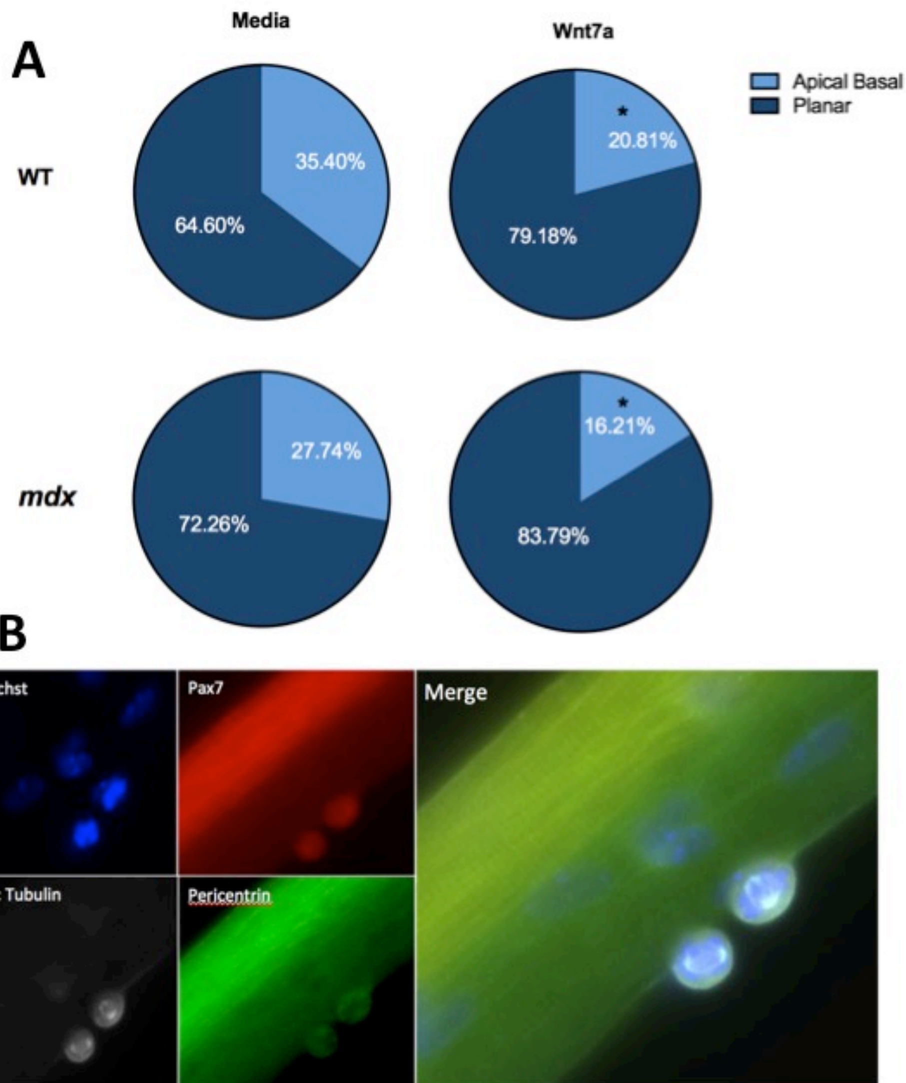

Figure 5: Wnt7a promotes the planar orientation of satellite cell division. (A) The proportions of cells with centrosome pairs poised to divide in either an apical-basal or planar orientation on *Myf5-Cre:R26R-YFP* myofibers fixed at 36h. (B) Representative images of dividing satellite cells on myofibers fixed at 36h. Image acquired at 630x magnification. * P < 0.05 as determined by student's t-test, compared to non-treated controls.

To establish if the increase in planar divisions correlates with an increase in symmetric divisions, *Myf5-Cre:R26R-YFP* myofibers were also fixed at 42h, the point at which activated SCs have completed their first round of division (Brun, Wang, and Rudnicki 2018). Wnt7a treatment resulted in a significant increase in the number of completed symmetric divisions by almost 4-fold in WT fibers and 2-fold in *mdx* fibers (Figure 6A). There was no change in the overall number of divisions or SCs between treatment groups and controls, suggesting that Wnt7a was not acting on overall cell proliferation but rather cell fate determination (Figure 6B). Here, we demonstrate the activity of Wnt7a by confirming its ability to promote the symmetric expansion of SCs, consistent with previous findings (Le Grand et al. 2009).

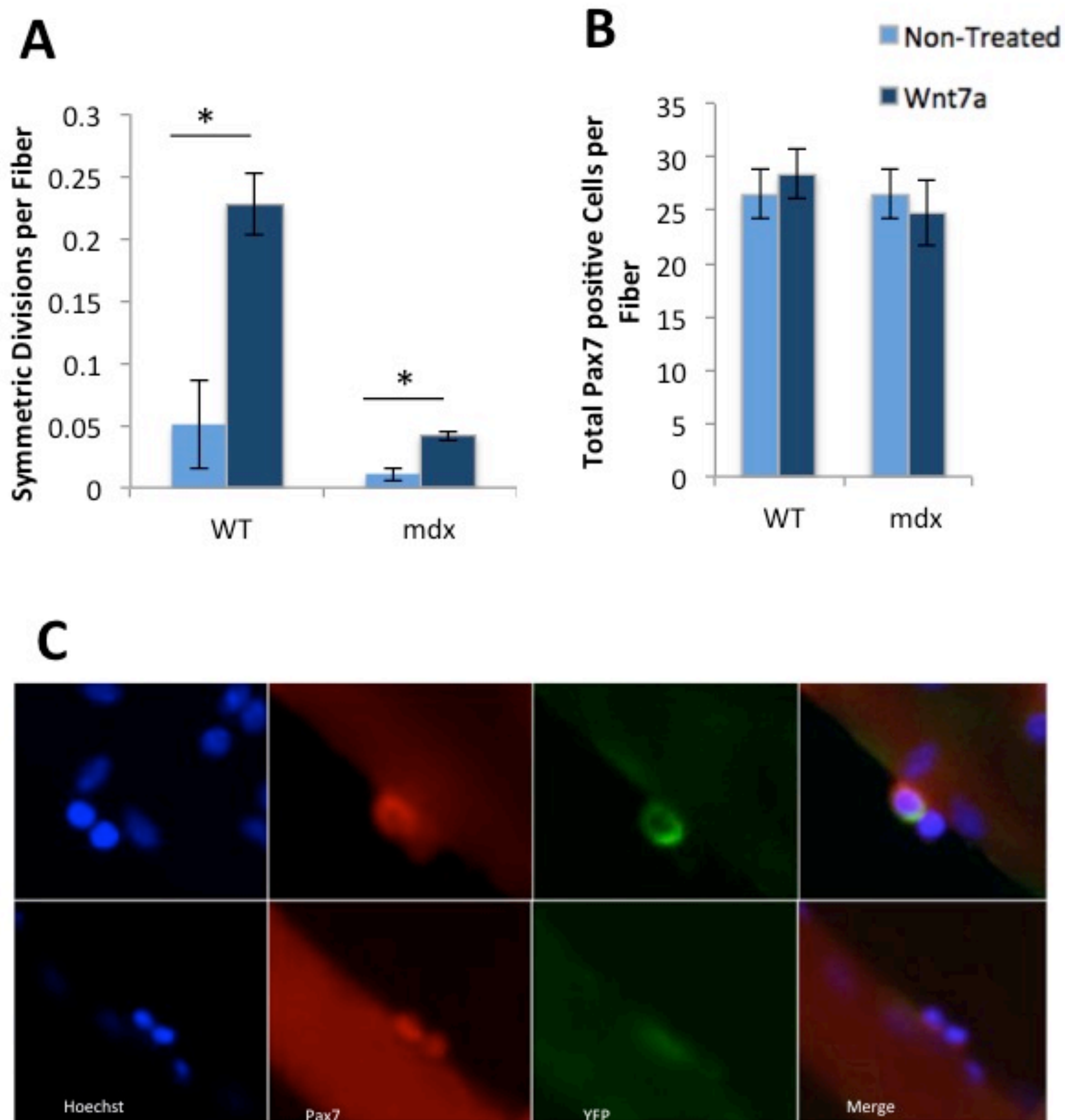

Figure 6: Wnt7a treatment drives SC symmetric expansion. (A) Wnt7a treatment promotes symmetric SC divisions in both WT and *mdx Myf5-Cre:R26R-YFP* myofibers following 42h of culture. (B) The total number of Pax7 positive cells per fiber on *Myf5-Cre:R26R-YFP* myofibers fixed at 42h. (C) Representative image of an asymmetric division (Top) and a symmetric division (Bottom). Both images were acquired at 200x magnification. Error bars represent ± SEM, * $P < 0.05$ as determined by student's t-test, compared to non-treated controls.

Though the influence of Wnt7a on satellite cell division has been well characterized, the effect of Wnt7a on myogenic differentiation has been less investigated. Given that Wnt7a treatment has been previously reported to ameliorate dystrophic symptoms in *mdx* mice, and *mdx* mice display decreased committed progenitors, we were interested in whether Wnt7a ameliorates DMD by promoting myogenic differentiation (von Maltzahn, Renaud, et al. 2012; Dumont et al. 2016). To establish the influence of Wnt7a treatment on SC differentiation, myofibers from WT and *mdx Myf5-Cre:R26R-YFP* mice were fixed after 72h of culture and stained for MyoG, an early muscle differentiation marker. Surprisingly, Wnt7a treated fibers had a 2-fold increase in MyoG expression in both WT and *mdx* mice, when compared to untreated controls (Figure 7A). No difference in the overall cell numbers was observed between Wnt7a treated fibers and control, suggesting Wnt7a was not influencing the rate of SC division.

To further establish the influence of Wnt7a on MyoG expression we performed both RT-qPCR and western blot analysis on WT myoblasts differentiated *in vitro* for 72h. RT-qPCR analysis revealed no differences in transcript levels of MyoG between Wnt7a treated and non-treated controls throughout differentiation. In contrast to mRNA expression, protein analysis via western blot had significant increases of MyoG protein levels during the first 24h of differentiation in Wnt7a treated cells (Figure 7D-E). These findings further suggest Wnt7a may promote translation of MyoG and further support a role for Wnt7a during myogenic differentiation.

Overall, we confirm the activity of Wnt7a by demonstrating its ability to promote symmetric SC division occurring in a planar orientation. Additionally, we

observed an increase in MyoG expression following Wnt7a treatment of both *in vitro* myoblast culture and *ex vivo* myofiber culture. These findings suggest Wnt7a may have an additional role in myogenic differentiation, which has yet to be defined.

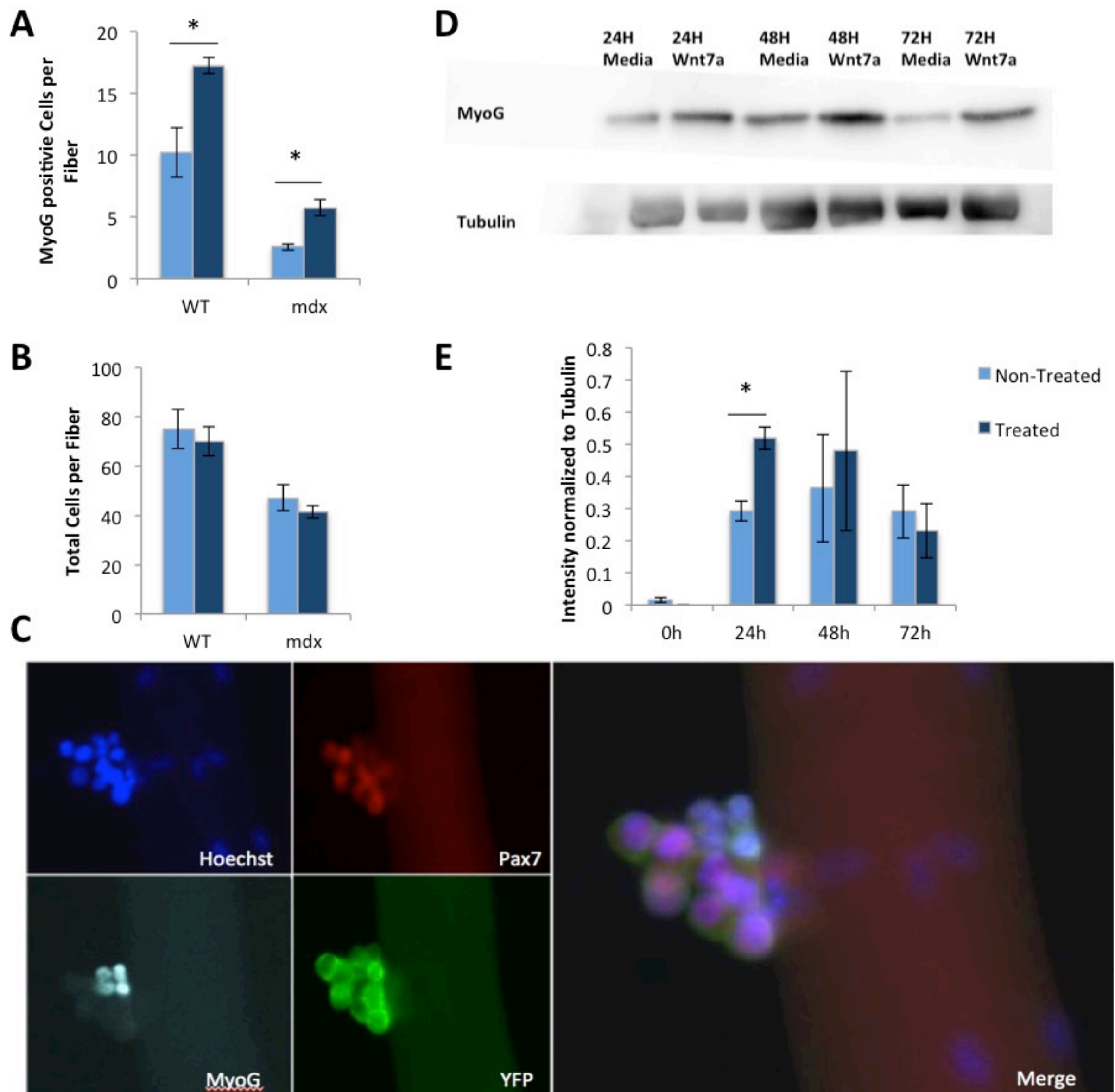

Figure 7: Wnt7a treatment promotes MyoG expression. (A) The number of MyoG postitive cells following Wnt7a treatment on both WT and *mdx Myf5-Cre:R26R-YFP* fixed after 72h of culture. (B) Total number of cells in *Myf5-Cre:R26R-YFP* fixed after 72h of culture. (C) Representative images of 72h *Myf5-Cre:R26R-YFP* myofibers. Image acquired at 630x magnification. (D) Western blot of MyoG throughout 72h of *in* vitro myoblast differentiation ± Wnt7a. (E) Quantification of MyoG western band intensity, normalized to Tubulin. Error bars represent ± SEM, * $P < 0.05$ as determined by student's t-test.

3.2 PCP Signaling Promotes Ciliogenesis During Myogenic Differentiation

Previous reports demonstrate that activation of the PCP pathway influences ciliogenesis (Heydeck, Zeng, and Liu 2009; Zeng, Hoover, and Liu 2010). Hh signaling and ciliogenesis are tightly linked; therefore, loss of ciliogenesis disrupts Hh signaling. For example, during myogenesis, loss of primary cilia is associated with decreased *Gli1* and *Ptch1* expression alongside defects associated with aberrant Hh signaling such as polydactyly (Kopinke et al. 2017). Additionally, disruption of cilia formation and subsequently Hh signal regulation have adverse effects during myogenic differentiation (Fu, Asp, Canter, and Dynlacht 2014). Given the influence PCP signaling has on ciliogenesis, and importance of Hh signaling during myogenic differentiation, we speculate that crosstalk between these pathways is responsible for Wnt7a-mediated MyoG expression.

To establish if Wnt7a treatment alters ciliogenesis during differentiation, we isolated myofibers from both WT and *mdx ARL13b-mcherry* mice. ARL13b is a protein that localizes to the primary cilia, allowing for the primary cilia to be visualized. Since cilia are not present in cycling cells, myofibers were treated with Wnt7a throughout culture and analyzed at 72h to allow cell cycle exit (Jimmy Kuang-Hsien Hu et al. 2012). Interestingly, 60% of cells in WT mice were ciliated while only 20% of cells were ciliated on *mdx* myofibers (Figure 8D). These findings demonstrate a cilia defect occurs in DMD, which may be associated with aberrant Hh signaling and subsequently contribute to disease progression. Conversely, no differences in primary cilia was observed between Wnt7a treated myofibers and non-treated controls, disagreeing with the notion that the PCP pathway influences

ciliogenesis (Heydeck, Zeng, and Liu 2009; Zeng, Hoover, and Liu 2010).

Ciliogenesis occurs transiently during myogenic differentiation; therefore it is likely that the full extent of cilia formation may not be observed in myofiber culture since we may not be quantifying an appropriate number of events. Thus, we evaluated cilia numbers in primary myoblasts isolated from the hindlimb of *ARL13b-mcherry* mice. Myoblasts were analyzed for the number of mononucleated ciliated cells at 6h, 12h, 24h, and 48h following the introduction into differentiation conditions and either the presence or absence of Wnt7a. Cells were stained with Pax7, ARL13b and α-Tubulin to mark SCs, cilia and myotubes respectively.

Overall, the number of mononucleated *ARL13b* positive cells increased throughout differentiation, agreeing with previous findings from C2C12 cells (Figure 8 A-C)(Fu, Asp, Canter, and Dynlacht 2014) . No significant differences in either the fusion or differentiation indexes were found between treated and non-treated groups (Figure 9). Notably, myoblasts treated with Wnt7a had increasingly more ciliated cells than non-treated controls from 12h to 48h of differentiation. These data demonstrate that Wnt7a treatment promotes primary cilia formation during *in vitro* differentiation. This is in concert with previous literature stating Wnt7a activation of the PCP pathway promotes ciliogenesis (Heydeck, Zeng, and Liu 2009; Zeng, Hoover, and Liu 2010).

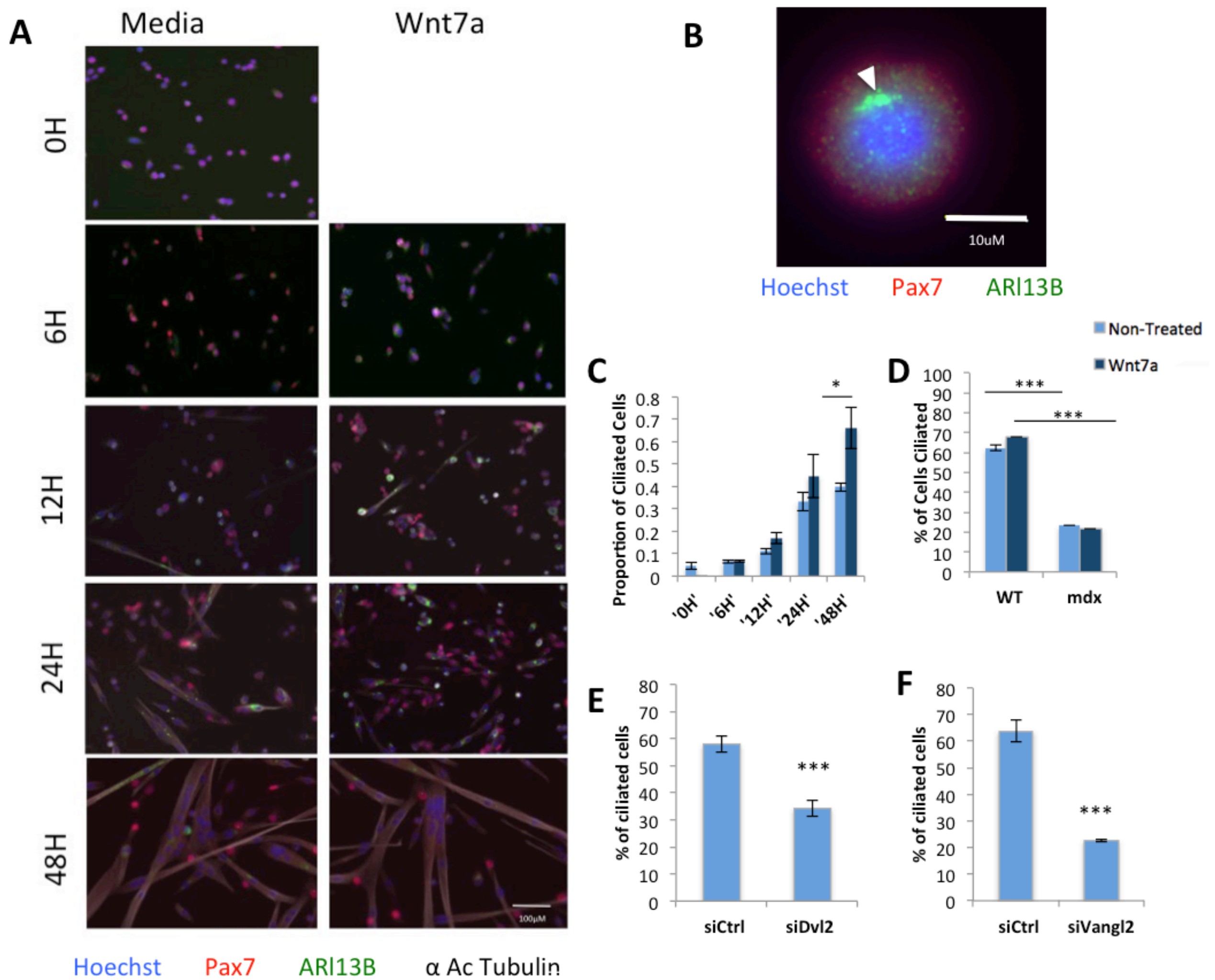

Figure 8: Wnt7a activation of PCP pathway influences ciliogenesis. (A) Representative images from *ARL13b-mCherry* myoblast differentiation, images were taken at 200x magnification. (B) A representative image of an ARL13b positive myoblast image taken at 630x magnification. Arrow indicates primary cilia as marked by ARL13b expression. (C) The proportion of mononucleated ciliated cells, as marked by ARL13b expression, throughout myoblast differentiation. (D) The proportion of Pax7 and ARL13b positive cells in both WT and *mdx ARL13b-mCherry* myofibers fixed at 72h. (E) *Arl13b-mCherry* myofibers fixed at 72h and analyzed for the number of primary cilia following knockdown of siDvl2 and (F) siVangl2. Error bars represent ± SEM, * $P < 0.05$, *** $P<0.001$ as determined by student's t-test.

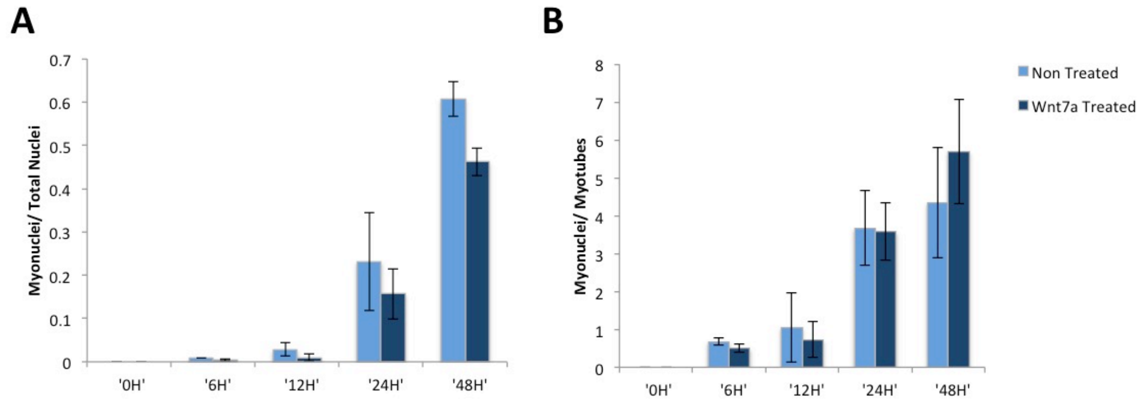

Figure 9: Differentiation of Wnt7a treated ARL-13b-mcherry myoblasts. (A) The differentiation index and (B) the Fusion index was calculated during 72h of myoblast differentiation ± Wnt7a (100ng/ml). Error bars represent ± SEM.

Given that *in vitro* differentiation assays demonstrated ciliogenesis changes with Wnt7a treatment, we wanted to confirm if PCP signaling influences ciliogenesis in myofibers. Since Wnt7a treatment on myofibers did not alter ciliogenesis likely due to the transient presence of cilia, we sought to knockdown PCP signaling as an alternative approach. We knocked down Wnt signaling in *ARL13b-mcherry* myofibers, using a siRNA targeted towards the core PCP protein Dvl2 *(siDvl2)*. Primary cilia numbers were then analyzed after 72h of culture while test conditions were blinded to the observer.

Myofibers treated with *siDvl2* had significantly less ciliated cells than those treated with scrambled controls, implicating an importance for Wnt signaling in ciliogenesis (Figure 8E). Dvl2 is critical for signal transduction of both canonical and non-canonical Wnt signaling therefore, to separate the effects of non-canonical signaling from that of canonical Wnt signaling, we also treated myofibers with a siRNA targeted towards Vangl2 (*siVangl2*), a PCP specific protein. Loss of Vangl2 resulted in a decreased proportion of ciliated cells similar to that observed following Dvl2 knockdown, indicating that the non-canonical PCP pathway and not canonical Wnt signaling influences ciliogenesis during *in vitro* differentiation (Figure 8F).

Here we demonstrate through myofiber culture that primary cilia formation is impaired in *mdx* mice. Additionally, exogenous Wnt7a treatment promotes ciliogenesis during *in vitro* differentiation, while inhibiting the PCP pathway impairs ciliogenesis of myogenic precursors. Taken together these data demonstrate that Wnt7a activation of the PCP pathway is required to promote ciliogenesis during adult muscle regeneration.

3.3 Ciliogenesis is Required for Asymmetric Division and Wnt7a Mediated Myog Expression

Hh signaling is an established regulator of cell cycle in both embryonic myogenesis and adult muscle regeneration. However, the role of Hh signaling during myogenic fate determination requires further investigations (Kim et al. 2011; Jaafar Marican, Cruz-Migoni, and Borycki 2016; Straface et al. 2009). In mouse embryos, the Gli transcription factors are reported to activate *Myf5* expression (Anne-Gaëlle Borycki et al. 1999). Since *Myf5* is differentially expressed during a SC asymmetric division and the Gli transcription factors target *Myf5,* we reasoned that Hh signaling is required during asymmetric SC division.

To characterize if Hh signaling is required during asymmetric divisions, we knocked down ciliogenesis which has the effect of reducing Hh signaling, in *Myf5-Cre:R26R-YFP* myofibers. We knocked down ciliogenesis with siRNAs targeting one of two intraflagellar transport (IFT) proteins required for cilia formation (*IFT20* and *IFT88*). Myofibers were fixed at 42h to determine the role of Hh signaling during SC division and at 72h to establish how Hh signaling influences differentiation. Myofibers were also co-treated with Wnt7a to establish if cilia are required for any Wnt7a mediated effects.

Knockdown of either *IFT20* or *IFT88* did not alter the number of symmetric divisions in myofibers fixed at 42h. Furthermore, knockdown of ciliogenesis did not interfere with Wnt7a driven symmetric SC expansion (Figure 10B). Conversely, asymmetric divisions were decreased following knockdown of either *IFT20* or *IFT88*. This decrease was amplified by the addition of exogenous Wnt7a (Figure

10A). These data are in concert with findings suggesting that proper Hh signal transduction is required for the regulation of *Myf5* expression, and subsequently asymmetric divisions (Anne-Gaëlle Borycki et al. 1999; Duprez, Fournier-Thibault, and Nicole Le Douarin 1998).

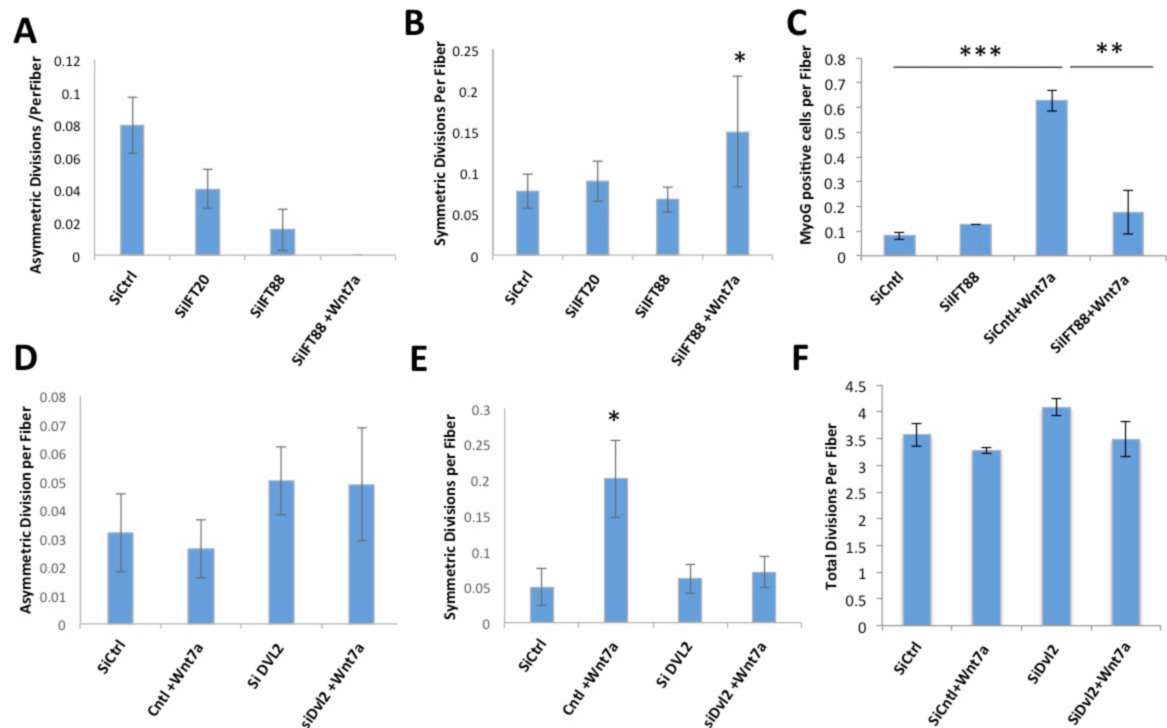

Figure 10: The roles of Hh and Wnt signaling during satellite cell division. Ciliogenesis was knocked down in *Myf5-Cre:R26R-YFP* myofibers using siRNAs targeted towards *IFT20* or *IFT88*. Myofibers were fixed at 42h and analyzed for (A) the number of asymmetric divisions, and (B) symmetric divisions. (C) *Myf5-Cre:R26R-YFP* myofibers fixed at 72h were analyzed for the number of MyoG positive cells, following knockdown of ciliogenesis. Wnt signaling in *Myf5-Cre:R26R-YFP* myofibers was knocked down using *siDvl2* and analyzed at 42h for (D) asymmetric divisions and (E) symmetric divisions. (F) Total number of divisions following treatment with *siDvl2*. Error bars represent ± SEM, * $P < 0.05$, ** $P<0.01$, *** $P<0.001$ as determined by student's t-test, compared to scrambled controls (*siCntl*) unless otherwise indicated.

To determine if impaired ciliogenesis affects Wnt7a mediated MyoG expression, *Myf5-Cre:R26R-YFP* myofibers were also analyzed after 72h of culture. Treatment with either *siIFT88* or *siIFT20* did not significantly alter the overall number of MyoG expressing cells (Figure 10C). Interestingly, ciliogenesis knockdown resulted in a 3.6 fold decrease in Wnt7a-mediated MyoG expression when compared to controls. No significant changes in overall cell numbers were observed between test groups. These data demonstrate that ciliogenesis is required for Wnt7a mediated MyoG expression, supporting the notion that crosstalk between the PCP and Hh signaling pathways are important for myogenic differentiation.

Given the role Hh signaling plays in asymmetric division we sought to determine if crosstalk between the PCP and Hh pathways occurs during SC proliferation. To establish this we knocked down Wnt signaling in *Myf5-Cre:R26R-YFP* myofibers using an siRNA for *Dvl2*. Myofibers were fixed at 42h and analyzed for numbers of asymmetric and symmetric divisions while conditions were blinded to the observer. Since *Dvl2* is also required for canonical Wnt signaling which has been reported previously to influence SC activation, the total number of divisions in each group was counted (Murphy et al. 2014). No significant changes in the total number of cell divisions was observed implicating that SC activation was not altered in this experiment (Figure 10F). Unsurprisingly, knockdown of *Dvl2* prevented Wnt7a driven symmetric expansion of SCs. Conversely, the number asymmetric divisions remained unchanged (Figure 10D-E). Since loss of cilia signaling, but not loss of Wnt signaling, altered asymmetric divisions these data indicate that crosstalk between these pathways does not occur during SC proliferation.

Our results indicate that active Hh signaling is required for asymmetric SC division, which is in concert with its ability to activate *Myf5*. In addition, Hh signaling seems to be required for Wnt7a mediated MyoG expression during SC differentiation, suggesting crosstalk between the PCP and Hh pathways is required for Wnt7a driven differentiation. Conversely, knockdown of Wnt signaling did not alter the proportion of asymmetric divisions suggesting that the PCP and Hh signaling pathways hold independent roles during SC proliferation.

3.4 Gli3 Expression Increases with Wnt7a Treatment During Early Differentiation

Gli3, is one of the main transcription factors involved in Hh signal transduction and has been suggested to be a key regulator of Hh signaling during muscle differentiation (Fu, Asp, Canter, and David 2014). Thus, we suspect Gli3 to be an important factor in the crosstalk between the PCP and Hh pathways. We sought to characterize any changes in mRNA expression of *Gli3* and of the Hh reporter *Gli1* during myoblasts differentiation following Wnt7a treatments. To establish any mRNA expression changes we performed RT-qPCR analysis. Expression of both *Gli1* and *Gli3* was decreased throughout the 72h of differentiation, agreeing with previous reports (Figure 11A-B) (Caroline Brun & Michael Rudnicki; unpublished) (Fu, Asp, Canter, and Dynlacht 2014). Treatment with Wnt7a increased the expression of *Gli3* after 24h of differentiation (Figure 11A). These findings agree with the notion that crosstalk between the PCP and Hh pathways act through regulation of *Gli3*. The expression of *Gli1* relies on activation by Gli2 and Gli3 making it a reporter of Hh signaling (Nozawa, Lin, and Chuang 2013). The expression of *Gli1*,

was not significantly altered following Wnt7a treatment (Figure 11B). The expression of *Gli3* and not *Gli1* suggests that Gli3 may be working in its repressor form to silence Hh signaling.

In addition to the Gli transcription factors, we also measured the expression of several Hh and PCP signaling targets. No significant differences were observed in the expression of *Myf5*, *MyoG*, *Intu, Fuz, Vangl2* or *Dvl2* (Figure 11C-H). Overall, we demonstrate that *Gli3* expression alongside several PCP components is increased during early differentiation following Wnt7a treatment. This may suggest roles for Gli3 during crosstalk between the Hh and PCP pathways. However, protein analysis is required to confirm these implications.

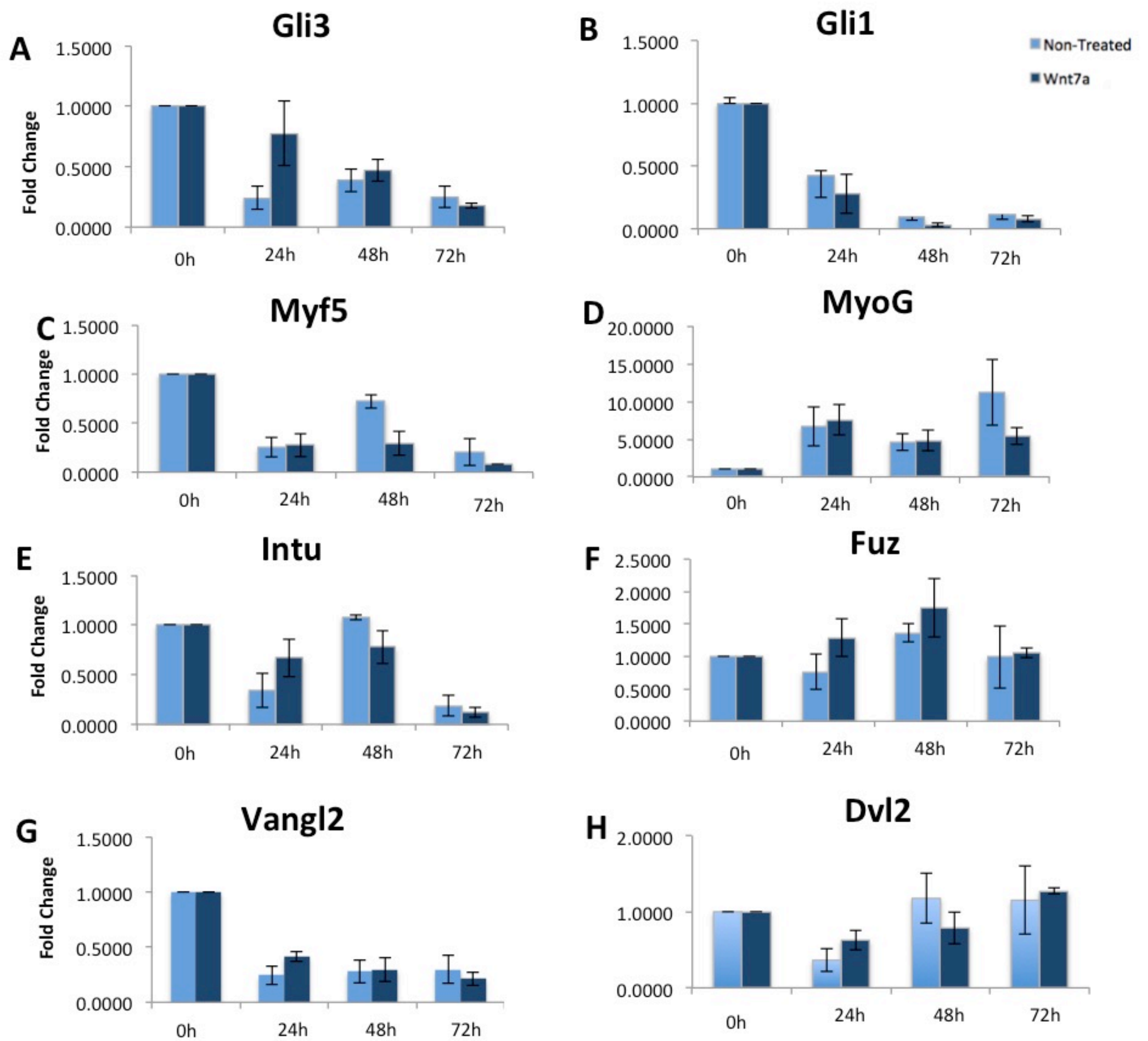

Figure 11: RT-qPCR of selected genes throughout 72h of myoblast differentiation ± Wnt7a (100ng/ml). Error bars represent ± SEM.

3.5 Fuzzy and Inturned as Candidates For Crosstalk

Having demonstrated that crosstalk between the PCP and Hh signaling pathways likely occurs to regulate later stages of muscle regeneration, we next sought to identify proteins acting as mediators for this crosstalk. The two downstream PCP effectors, Intu and Fuz are known to mediate crosstalk between the PCP and Hh signaling pathways during embryonic myogenesis (Heydeck, Zeng, and Liu 2009; Zeng, Hoover, and Liu 2010). Given that muscle regeneration closely resembles myogenesis we selected Intu and Fuz as our primary candidates. Since the PCP pathway is reported to act on Hh signaling through ciliogenesis, we knocked down either Intu or Fuz in *ARL13b-mcherry* myofibers and assessed primary cilium numbers. Myofibers in which either Intu or Fuz was knocked down had over a 2-fold reduction in *ARL13b* positive cells compared to controls (Figure 12A-B). These findings suggest *Intu* and *Fuz* hold influential roles over ciliogenesis.

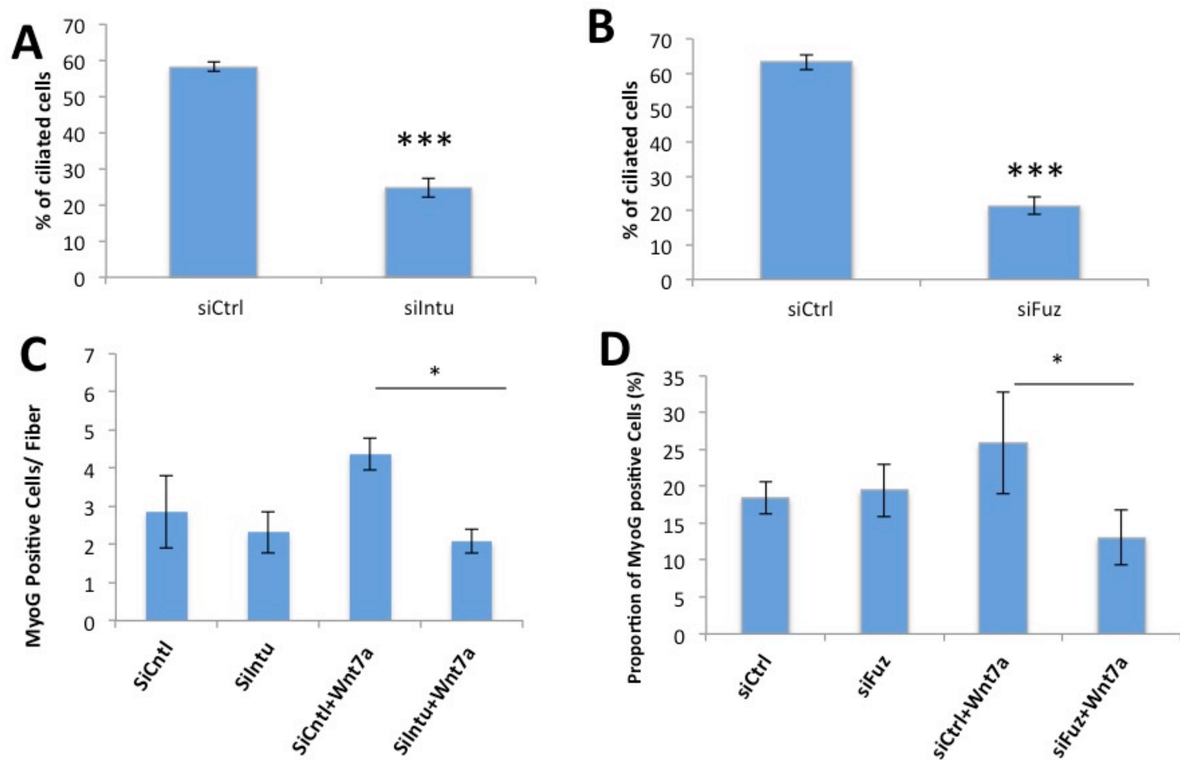

Figure 12: A role for Fuzzy and Inturned during PCP and Hh signaling crosstalk. (A) Primary cilia numbers in *ARL13b-mcherry* myofibers fixed at 72h following siRNA knockdown of *Intu* and (B) *Fuz.* (C) The number of MyoG positive cells on *Myf5-Cre:R26R-YFP* myofibers fixed at 72h following knockdown of *Intu* and (D) *Fuz*. Error bars represent ± SEM, * $P < 0.05$, ** $P<0.01$, *** $P<0.001$ as determined by student's t-test, compared to *siCntl* unless otherwise indicated.

Since we suspect crosstalk between the PCP and Hh pathways drives Wnt7a-mediated MyoG expression, we were interested in determining the effect of Intu and Fuz knockdown on MyoG expression. To determine this *Myf5-Cre:R26R-YFP* myofibers were treated with either *siIntu* or *siFuz* and analyzed at 72h for MyoG expression. Overall, knockdown of either *Intu* or *Fuz* prevented Wnt7a-mediated MyoG expression, agreeing with the notion that Intu and Fuz mediate crosstalk between the PCP and Hh pathways (Figure 12C-D).

In all, knockdown of *Intu* and *Fuz* was associated with a reduction in both ciliogenesis and Wnt7a driven MyoG expression. These findings support the notion that Intu and Fuz mediate the crosstalk between PCP and Hh signaling pathways. In addition these findings agree with reports from embryogenesis, suggesting crosstalk between the PCP and Hh pathways during adult muscle regeneration occur in a similar fashion to that of embryogenesis.

Chapter4: Discussion

Understanding what mechanisms regulate SC fate is crucial for the discovery of new therapeutic avenues for DMD. Both the PCP and Hh signaling pathways have been identified to be key regulators of SC proliferation and differentiation. However, crosstalk between these pathways has not yet been investigated in the context of muscle regeneration. Previous literature shows that crosstalk between these pathways regulates embryonic myogenesis, a process that muscle regeneration closely resembles. In this study, we have identified that crosstalk between the PCP and Hh pathways promote myogenic differentiation. Consistent with findings in embryonic myogenesis, activation of the PCP pathway in adult muscle regeneration also promotes primary cilia formation. PCP effectors Intu and Fuz, were found to be responsible for mediating crosstalk between the PCP and Hh pathways

4.1 Wnt7a Influences Myogenin Expression

Our lab has previously demonstrated that Wnt7a acts through the PCP pathway to drive the symmetric expansion of SCs, however the consequences of this event in the later stages of muscle regeneration remain unclear (Le Grand et al. 2009). Surprisingly, sustained Wnt7a treatment during differentiation increases MyoG expression, suggesting that activation of the PCP pathway promotes muscle differentiation.

In paraxial mesoderm explants, Tajbakhsh and colleagues demonstrate Wnt7a to promote the expression of *MyoD*, which subsequently initiates myogenic

differentiation (Tajbakhsh et al. 1998). These findings support the notion that Wnt7a treatment promotes myogenic differentiation. Additionally, the induction of the myogenic lineage in Tajbakhsh's report occurred independently of Myf5 activation, suggesting the possibility of alternative differentiation routes (Tajbakhsh et al. 1998).The ability of SCs to differentiate while lacking either MyoD or Myf5 demonstrate the compensatory roles between these proteins, and explains why in this current study we observe increases in MyoG expression without increases in Myf5 expression (A.Rudnicki et al. 1992; Megeney et al. 1996).

Contrarily, Von Maltzahn and colleagues show that overexpression of Wnt7a during the differentiation of C2C12 cells increases myofiber hypertrophy without altering MyoG expression(von Maltzahn, Bentzinger, and Rudnicki 2012). However, methodological differences such as the chosen delivery mechanism and timing for Wnt7a treatments could explain discrepancies between our studies. One could address these issues by utilizing the Wnt7a overexpression vector, as used by Von Maltzahn, in the context of this current study.

Additionally, Le Grand and colleagues demonstrate that early loss of PCP signaling through siRNA knockdown of *Vangl2* increases MyoG expression (Le Grand et al. 2009). However, this early loss of PCP signaling not only affects differentiation of SCs but also their proliferation and specifically, SC self-renewal. This compromises the balance between SC differentiation and self-renewal, which is critical for proper muscle homeostasis. For example, loss of Notch signaling through the knockout of the RBP-J transcription factor increases the number of *MyoG* expressing cells (Vasyutina et al. 2007). However, this increase was accompanied by

a significant loss of myogenic progenitors, which later was associated with muscle hypotrophy.

The consequence of lost stem cell self-renewal, in the context of Wnt7a signaling is highlighted in neurogenesis. In this case, the genetic ablation of Wnt7a lead to an increase in neuronal differentiation, and a reduction in the stem cell pool (Qiuhao Qu et al. 2013). This was associated with decreases in cyclin D1 expression, a protein critical for cell cycle progression. Though this study reported Wnt7a to work through the canonical pathway as marked by activation of β-catenin, cyclin D1 may also be activated by the PCP activated protein, Rock[62]. This is supported by findings in C2C12 cells, where loss of Rock promotes differentiation as marked by increases in MyoG expression[63]. Therefore, it is likely that the loss of PCP signaling during early stages of muscle regeneration could increase differentiation but likely at the expense of the resident SC pool compromising future muscle regeneration.

Due to the complexity of Wnt signaling, the delivery mechanism, the timing of Wnt treatments and the alternative effects of lost signaling all contribute to the observed pheotype. It is crucial to consider these factors when assessing the effects of Wnt in the literature. This has complicated the translation of Wnt proteins into clinical therapies and should be addressed in future Wnt focused investigations.

4.2 Crosstalk Between Hh and PCP Signaling is Responsible for Wnt7a-Mediated MyoG Expression.

Early observations showing that Shh and Wnt7a co-localize within the paraxial mesoderm during embryogenesis suggest that the PCP and Hh pathways might interact during the induction of myogenesis (Tajbakhsh et al. 1998). Indeed, the expression of MyoD is significantly higher in paraxial mesoderm explants, which express both Shh and Wnt7a compared to explants that only express one of these ligands (Tajbakhsh et al. 1998). More recent findings demonstrate crosstalk between the PCP and Hh signaling pathways is linked to the regulation of ciliogenesis (Heydeck, Zeng, and Liu 2009; Zeng, Hoover, and Liu 2010). However, whether this crosstalk is conserved throughout adult muscle regeneration has not yet been considered.

We show that Wnt7a treatment promotes cilia assembly during myogenic differentiation. Moreover, the knockdown of PCP components *Dvl2* and *Vangl2* decrease ciliation, further supporting a role for PCP signaling during ciliogenesis. In addition, loss of cilia formation impairs Wnt7a-mediated MyoG expression suggesting that crosstalk between the PCP and Hh pathway is required.

Reports that PCP effectors Intu, and Fuz, are required, in part, for cilia formation during myogenesis are consistent with our findings that loss of these proteins reduce myogenesis (Heydeck, Zeng, and Liu 2009; Zeng, Hoover, and Liu 2010). In addition, other PCP components such as Dvl2, and Vangl2 have been observed to localize at the basal body of the primary cilium, which is in congruence with potential crosstalk between these pathways (Hamblet 2002; Z et al. 2001). In

fact, *Dvl2*$^{-/-}$ and *Vangl2*$^{-/-}$ mice have been reported to exhibit reduced ciliogenesis, and aberrant Hh signaling as marked by neural tube closure defects during development (Luo et al. 2001; NS et al. 2002). However, it is difficult to establish individual roles for *Dvl2* and *Vangl2* during these studies, since their activation is required for the downstream activation of Intu and Fuz.

It is likely Intu and Fuz influence primary cilia formation through regulating the cytoskeleton. Indeed, in *Xenopus* models, loss of Intu and Fuz is associated with disrupted microtubule organization (Park, Haigo, and Wallingford 2006). Additionally, during PCP signaling, Intu and Fuz are required to re-organize microtubules to promote convergent extension in development. Given that the basal body positioning of primary cilia is regulated by microtubule organization it is likely that Intu and Fuz regulate cilia formation through mediating microtubule organization (Park, Haigo, and Wallingford 2006).

4.3 Hh Signaling and PCP Pathway Hold Independent Roles in SC Proliferation

It is well established that stimulation of the PCP pathway promotes the symmetric expansion of SCs, but the role of Hh signaling on SC proliferation has yet to be elucidated (Kuang et al. 2007). Here, we examined the effect of Hh signaling in SCs through the blockade of ciliogenesis. In contrast to the PCP pathway, loss of ciliogenesis, decreases the proportion of asymmetric divisions (Figure 8). Intriguingly, during myogenesis Gli2 and Gli3 bind to *Myf5* to induce myogenic commitment (Anne-Gaëlle Borycki et al. 1999). Thus, it is likely Hh signaling is required during an asymmetric division, to activate *Myf5* transcription in the

committed progenitor. This effect seems to be independent of PCP signaling, as loss of PCP signaling did not alter the number of asymmetric divisions, implicating crosstalk between these two pathways only during later stages muscle regeneration.

The lack of crosstalk between PCP and Hh signaling during SC proliferation may arise from the timing at which cilia re-assembly occurs. Primary cilium are typically present during quiescence and disassemble during entry into the cell cycle (Jaafar Marican, Cruz-Migoni, and Borycki 2016). For example, blockade of SC proliferation via *CDK4/6* inhibition prevents primary cilium disassembly. Conversely, the removal of *CDK4/6* inhibitors allows SCs re-enter the cell cycle and primary cilia to disassemble (Jaafar Marican, Cruz-Migoni, and Borycki 2016). Conversely, studies in the mouse embryonic fibroblast cell line, NIH-3T3, demonstrate that prolonging cilia disassembly by blocking microtubule rearrangements with Cytochalasin D, delayed cell cycle entry (Kim et al. 2011). In general, the absence of the primary cilium, and subsequently Hh signaling machinery would allow for the PCP pathway to act independently of Hh signaling during SC proliferation.

On could elucidate the roles of Hh and PCP signaling during proliferation by considering the timing at which the Shh and Wnt7a ligands are expressed following injury. Typically Shh expression peaks around day 3 post injury while Wnt7a is not detected until day 6 (Le Grand et al. 2009; Straface et al. 2009). Considering active Hh signaling promotes cell cycle while, PCP signaling promotes SC expansion, it is likely that these ligands are transiently co-expressed. This co-expression is likely to

occur late in SC proliferation when Wnt7a is promoting SC pool expansion and just prior to cilia assembly, during cell cycle exit.

4.4 A Potential Role for Dystrophin in Cilia-Mediated Hh Signaling

Intriguingly, *mdx* mice have much lower proportions of ciliated SCs compared WT mice, suggesting that DMD might also be a ciliopathy. Given that Hh signaling is linked to ciliogenesis, it is likely that Hh signaling is also misregulated in DMD. Preliminary data from our laboratory supports this, as *mdx* myoblasts display reduced transcript expression of Hh targets including, *Gli1, Ptch1* and *Myf5* (Caroline Brun & Michael Rudnicki unpublished). Furthermore, following cardiotoxin injury, *Gli1* expression in murine hindlimb is significantly decreased in comparison to that of WT mice (Piccioni et al. 2014). *Gli1* expression requires the activation of the Hh pathway through Gil2 and Gli3. This reported decrease in Gli1 expression represents decreased Hh signaling. These findings further support the notion of aberrant Hh signaling in DMD. However, the mechanism of how loss of dystrophin alters Hh signaling has yet to be investigated.

Dysregulated Hh signaling following loss of dystrophin might be explained by impaired mitotic spindle formation as a result of dystrophin deletion. Compromised spindle formation has been shown to prolong and reduce the number of successful SC divisions in DMD (Dumont et al. 2016). Considering ciliogenesis and subsequently Hh signaling is tightly tied to cell cycle progression, prolonged divisions may affect the SC's time-sensitive response to Hh signals. This is highlighted during the asymmetric division of neural stem cells, where cilia form

asynchronously on the resulting daughter cells (Anderson and Stearns 2009). Since Hh signaling requires formed cilia, it can be speculated that the daughter cell that undergoes ciliogeneisis first will respond to the Shh signal differentially than its sister cell. Therefore, prolonged divisions may interfere with this, potentially leading to aberrant Hh signaling.

Alternatively, dysregulated Hh signaling following loss of dystrophin may be due to disrupted cell polarity. In SCs, dystrophin is required to establish the Par polarity complex. One member of this complex, Pard3, has been associated with ciliogenesis (Dumont et al. 2016). For example, Hong and colleagues demonstrated that loss of Pard3 in developing zebra fish, results defects in the motile cilia which emerge from epithelial cells lining the neural tube (Hong, Jayachandran, and Brewster 2010). These findings were associated with ciliary defects such as mislocalization of ciliary direction (Hong, Jayachandran, and Brewster 2010).

Studies in canine epithelial cells have also reported delayed ciliogenesis following loss of Pard3, further emphasizing its role in cilia formation (Sfakianos et al. 2007). These findings were attributed to interaction between Pard3 and Kinesin-2, a intraflagellar transport protein critical for cilia growth(Sfakianos et al. 2007). Given the requirement of Pard3 for proper cilia localization and its role during asymmetric division, it could be hypothesized that mislocalization of Pard*3* during asymmetric SC divisions results in decreased cilia formation in DMD.

4.5 Suspected Role for Gli3 During PCP and Hh Crosstalk

The ability of Gli3 to act as either a transcriptional activator or repressor makes it a crucial regulator of Hh signaling. Thus, to better understand crosstalk between the PCP and Hh pathways, it is necessary to determine the role of Gli3 during muscle differentiation. Increased Gli3 expression following Wnt7a treatment suggests it plays a role in PCP and Hh crosstalk (Figure 11). In addition, preliminary data from our lab demonstrates that a muscle-specific inducible knockout of *Gli3* leads to decreased MyoG expression during muscle regeneration (Caroline Brun & Michael Rudnicki; unpublished). These data support the notion that Gli3 plays roles in the regulation of muscle differentiation.

The role of cilia in regards to Gli3 processing has yet to be elucidated. However, the localization of Gli3 processing proteins to the cilium basal body indicates that cilia could be required to recruit the proteins required for Gli3 cleavage. (Jaafar Marican, Cruz-Migoni, and Borycki 2016; Walsh and Perlman 1997). Impaired Gli3 processing in C2C12 cells following the blockade of ciliogenesis supports this. These results were associated with impaired muscle regeneration suggesting that the transient reassembly of the primary cilium prior to differentiation is required to convert Gli3 to its repressor form(Fu, et al. 2014). As well, this implied that silencing of Hh signaling via the Gli3 repressor is required for myogenic differentiation.

To differentiate, cells must exit the cell cycle prior to fusing and forming myofibers (Walsh and Perlman 1997). Considering that several targets of Gli3 are involved in cell cycle progression, it is speculated that the Gli3 repressor form could

prevent cell cycle continuation allowing for muscle differentiation (Wilson et al. 2012). Additionally, through chromatin immunoprecipitation (ChIP) experiments MyoG has been reported to target genes associated with cell cycle exit (Straface et al. 2009; QC et al. 2012; K1 and FJ. 2013). This is confirmed in proliferating myoblasts, where ectopic expression of MyoG leads to cell cycle arrest (Liu et al. 2012). Therefore, Gli3 might act to repress genes promoting cell cycle progression, while MyoG acts to activate genes involved in cell cycle exit.

It is important not to rule out the possibility that Gli3 may promote the expression of MyoG or other differentiation genes either indirectly by silencing MyoG inhibitors or directly in its activator form. The ability of Gli3 to bind to other MRFs agrees with this notion since the MRFs are similarly regulated (Fu, Asp, Canter, and David 2014; Straface et al. 2009). In this current study we did not see a loss of MyoG expression following blockade of ciliogenesis, suggesting the role of Gli3 may be dispensable in specific contexts. This further emphasizes the importance of confirming the role of Gli3 in our model.

Considering the potential roles of Gli3 repressor with the findings of this study, we propose that during muscle differentiation, Wnt7a action of the PCP pathway results in the downstream activation of Intu and Fuz, which in turn promotes ciliogenesis. Transient ciliogenesis prior to differentiation allows for cell cycle exit and Gli3 processing. Once in its repressor form, Gli3 silences Hh signaling, allowing muscle differentiation, as marked by increased MyoG expression (Figure 13).

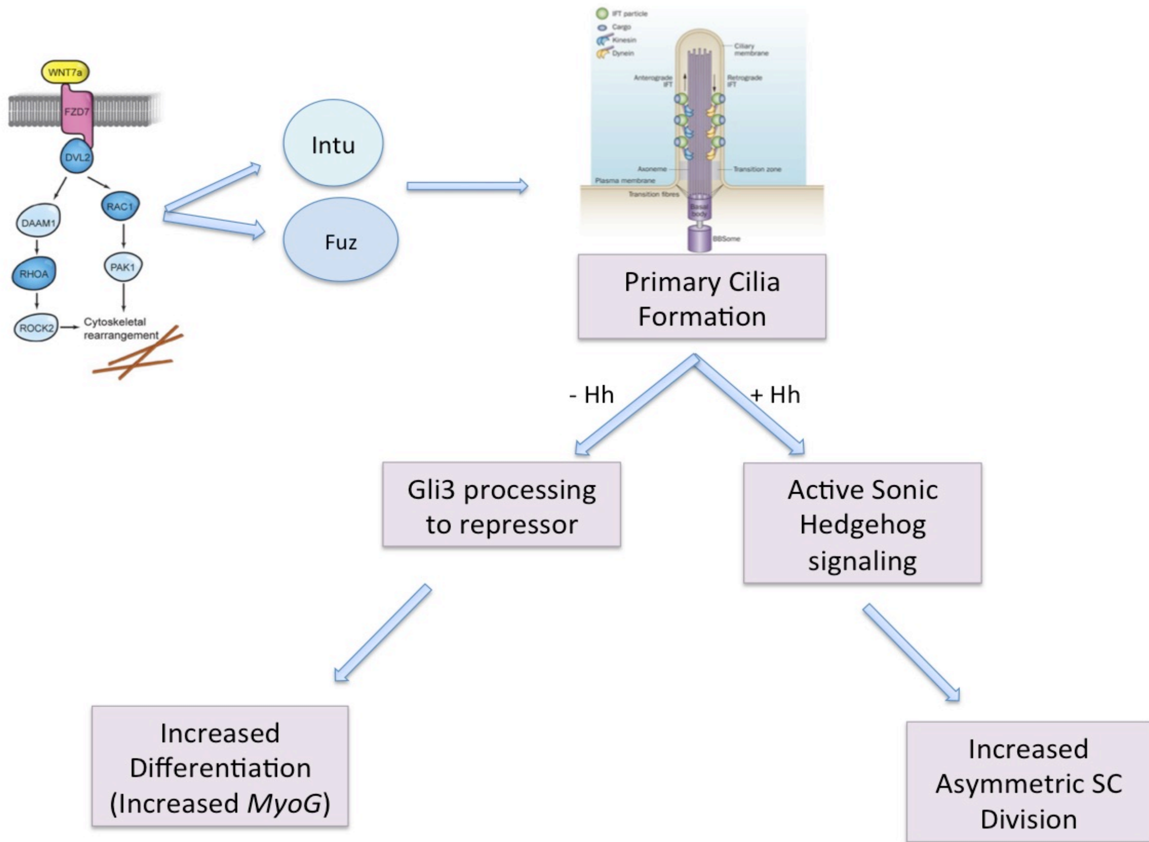

Figure 13: A schematic of the proposed crosstalk between PCP and Hh signaling during differentiation. Wnt7a binding to Fzd7 initiates PCP pathway and activates the downstream effectors Intu and Fuz, which promote the formation of the primary cilia. In the presence of the Hh ligand, Hh signaling is active and may act to promote asymmetric SC division. Conversely, in the absence of the Hh ligand Gli3 is processed to a repressor form to turn off Hh target genes and promote myogenic differentiation as marked by increased MyoG expression.

Chapter 5: Future Directions

Based on previous literature, we suspect Gli3 to be required for the crosstalk between the PCP and Hh signaling. Thus, future investigations should focus on elucidating the role of Gli3 during this crosstalk. One approach to completing this would be to preform protein analysis on differentiated myoblasts to determine if Wnt7a treatment alters levels of Gli3 activator and repressor forms. Given our proposed model, we predict Wnt7a treatment would increase the proportion of the Gli3 repressor. Alternatively, myofibers isolated from Gli3 floxed mice would allow us to analyze if Wnt7a treatment is able to drive MyoG expression in the absence of Gli3.

A second area of research that could stem from this project is to elucidate the role of dystrophin in Hh signaling. We demonstrate a primary cilia defect in *mdx* mice, and further more preliminary data suggest this is accompanied by aberrant Hh signaling (Caroline Brun & Michael Rudnicki unpublished). It is speculated that Pard3 mislocalization caused by the loss of dystrophin impairs ciliogenesis, which would disrupt Hh signaling. This could be investigated by knocking out Pard3 to determine if this affects ciliogenesis in a muscle setting. Additionally, future studies could look at targeting Hh signaling in DMD therapies. This could be achieved with drugs such as Purmorphamine, an agonist for smoothened, the Hh receptor.

Conclusions

In this study we demonstrate that crosstalk between the PCP and Hh signaling pathway promotes muscle differentiation, while each pathway holds individual roles in SC proliferation. Furthermore, we provide evidence suggesting the PCP effectors Intu and Fuz mediate crosstalk between these pathways similarly to reports in myogenesis. We suspect Gli3 in its repressor form is required for signaling transduction during this crosstalk however, further investigations are require to confirm this. Overall, understanding how crosstalk between the PCP and Hh signaling pathways provides insight on the regulation of SC differentiation and may lead to small molecule drug therapies for the treatment of Duchenne muscular dystrophy.

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